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<p>(21) International Application Number: PCT/US95/06846 (22) International Filing Date: 25 May 1995 (25.05.95) (30) Priority Data: 08/249,322 26 May 1994 (26.05.94) US (71) Applicants: LUDWIG INSTITUTE FOR CANCER RESEARCH [US/US]; 1345 Avenue of the Americas, New York, NY 10105 (US). CAMBRIDGE NEUROSCIENCE [US/US]; One Kendall Square, Cambridge, MA 02139 (US). (72) Inventors: GOODEARL, Andrew, David; 45 Blacketts Wood Drive, Chorleywood, Hertfordshire WD3 5PY (GB). STROOBANT, Paul; 52A Cecile Park, Crouch End, London N8 9AS (GB). MINGHETTI, Luisa; Via Stradello, 22, I-48012 Bagnacavallo (IT). WATERFIELD, Michael; Chantemerle, Speen Lane, Speen Newberry, Berkshire RG13 1RN (GB). MARCHIONNI, Mark; 24 Twin Circle Drive, Arlington, MA 02174 (US). CHEN, Maio, Su; 65 Decatur Street, Arlington, MA 02174 (US). HILES, Ian; 91 Riding House Street, London W1P 8BT (GB).</p>	<p>(74) Agent: PASQUALINI, Patricia, A.; Felfe &amp; Lynch, 805 Third Avenue, New York, NY 10022 (US).  (81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, JP, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MX, NO, NZ, PL, RO, RU, SI, SK, TJ, TT, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: GLIAL MITOGENIC FACTORS, THEIR PREPARATION AND USE  (57) Abstract  Disclosed is the characterization and purification of DNA encoding a numerous polypeptides useful for the stimulation of glial cell (particularly, Schwann cell) mitogenesis and treating glial cell tumors. Also disclosed are DNA sequences encoding novel polypeptides which may have use in stimulating glial cell mitogenesis and treating glial cell tumors. Methods for the synthesis, purification and testing of both known and novel polypeptides for their use as both therapeutic and diagnostic aids in the treatment of diseases involving glial cells are also provided. Methods are also provided for the use of these polypeptides for the preparation of antibody probes useful for both diagnostic and therapeutic use in diseases involving glial cells.</p>		

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## GLIAL MITOGENIC FACTORS, THEIR PREPARATION AND USE

Cross Reference to Related Applications

This application is a continuation-in-part of Serial No. 08/036,555, filed March 24, 1993, Serial No. 07/965,173, filed October 23, 1992, Serial No. 07/940,389, filed  
5 September 3, 1992, Serial No. 07/907,138, filed June 30, 1992 and Serial No. 07/863,703, filed April 3, 1992.

Background of the Invention

This invention relates to polypeptides found in vertebrate species, which polypeptides are mitogenic growth  
10 factors for glial cells, including Schwann cells. The invention is also concerned with processes capable of producing such factors, and the therapeutic application of such factors.

The glial cells of vertebrates constitute the  
15 specialized connective tissue of the central and peripheral nervous systems. Important glial cells include Schwann cells which provide metabolic support for neurons and which provide myelin sheathing around the axons of certain  
20 peripheral neurons, thereby forming individual nerve fibers. Schwann cells support neurons and provide a sheath effect by forming concentric layers of membrane around adjacent neural axons, twisting as they develop around the axons. These myelin sheaths are a susceptible element of many nerve  
25 fibers, and damage to Schwann cells, or failure in growth and development, can be associated with significant demyelination or nerve degeneration characteristic of a number of peripheral nervous system diseases and disorders. In the development of the nervous system, it has become  
30 apparent that cells require various factors to regulate their division and growth, and various such factors have

been identified in recent years, including some found to have an effect on Schwann cell division or development.

Thus, Brockes et al., *inter alia*, in J.

Neuroscience, 4 (1984) 75-83 describe a protein growth  
5 factor present in extracts from bovine brain and pituitary  
tissue, which was named Glial Growth Factor (GGF). This  
factor stimulated cultured rat Schwann cells to divide  
against a background medium containing ten percent fetal  
calf serum. The factor was also described as having a  
10 molecular weight of 31,000 Daltons and as readily  
dimerizing. In Meth. Enz., 147 (1987), 217-225, Brockes  
describes a Schwann cell-based assay for GGF.

Brockes et al., *supra*, also describes a method of  
purification of GGF to apparent homogeneity. In brief, one  
15 large-scale purification method described involves  
extraction of the lyophilized bovine anterior lobes and  
chromatography of material obtained thereby using NaCl  
gradient elution from CM cellulose. Gel filtration is then  
carried out with an Ultrogel column, followed by elution  
20 from a phosphocellulose column, and finally, small-scale SDS  
gel electrophoresis. Alternatively, the CM-cellulose  
material was applied directly to a phosphocellulose column,  
fractions from the column were pooled and purified by  
preparative native gel electrophoresis, followed by a final  
25 SDS gel electrophoresis.

Brockes et al. observed that in previously reported  
gel filtration experiments (Brockes et al., J. Biol. Chem.  
255 (1980) 8374-8377), the major peak of growth factor  
activity was observed to migrate with a molecular weight of  
30 56,000 Daltons, whereas in the first of the above-described  
procedures activity was predominantly observed at molecular

weight 31,000. It is reported that the GGF dimer is largely removed as a result of the gradient elution from CM-cellulose in this procedure.

Benveniste et al. (PNAS, 82 (1985), 3930-3934)  
5 describes a T lymphocyte-derived glial growth promoting factor. This factor, under reducing conditions, exhibits a change in apparent molecular weight on SDS gels.

Kimura et al. (Nature, 348 (1990), 257-260)  
describes a factor they term Schwannoma-derived growth  
10 factor (SDGF) which is obtained from a sciatic nerve sheath tumor. The authors state that SDGF does not stimulate the incorporation of tritium-labelled TdR into cultured Schwann cells under conditions where, in contrast, partially  
purified pituitary fraction containing GGF is active. SDGF  
15 has an apparent molecular weight of between 31,000 and 35,000.

Davis and Stroobant (J. Cell. Biol., 110 (1990),  
1353-1360) describe the screening of a number of candidate  
mitogens. Rat Schwann cells were used, the chosen candidate  
20 substances being examined for their ability to stimulate DNA synthesis in the Schwann cells in the presence of 10% FCS (fetal calf serum), with and without forskolin. One of the factors tested was GGF-carboxymethyl cellulose fraction (GGF-CM), which was mitogenic in the presence of FCS, with  
25 and without forskolin. The work revealed that in the presence of forskolin, inter alia, platelet derived growth factor (PDGF) was a potent mitogen for Schwann cells, PDGF having previously been thought to have no effect on Schwann cells.

30 Holmes et al. Science (1992) 256: 1205 and Wen et al. Cell (1992) 69: 559 demonstrate that DNA sequences which

encode proteins binding to a receptor (p185<sup>erbB2</sup>) are associated with several human tumors.

The p185<sup>erbB2</sup> protein is a 185 kilodalton membrane spanning protein with tyrosine kinase activity. The protein  
5 is encoded by the erbB2 proto-oncogene (Yarden and Ullrich Ann. Rev. Biochem. 57: 443 (1988)). The erbB2 gene, also referred to as HER-2 (in human cells) and neu (in rat cells), is closely related to the receptor for epidermal growth factor (EGF). Recent evidence indicates that  
10 proteins which interact with (and activate the kinase of) p185<sup>erbB2</sup> induce proliferation in the cells bearing p185<sup>erbB2</sup> (Holmes et al. Science 256: 1205 (1992); Dobashi et al. Proc. Natl. Acad. Sci. 88: 8582 (1991); Lupu et al. Proc. Natl. Acad. Sci. 89: 2287 (1992)). Furthermore, it is  
15 evident that the gene encoding p185<sup>erbB2</sup> binding proteins produces a number of variably-sized, differentially-spliced RNA transcripts that give rise to a series of proteins, which are of different lengths and contain some common peptide sequences and some unique peptide sequences. This  
20 is supported by the differentially-spliced RNA transcripts recoverable from human breast cancer (MDA-MB-231) (Holmes et al. Science 256: 1205 (1992)). Further support derives from the wide size range of proteins which act as (as disclosed herein) ligands for the p185<sup>erbB2</sup> receptor (see below).

25

#### Summary of the Invention

In general the invention provides methods for stimulating glial cell (in particular, Schwann cell and glia of the central nervous system) mitogenesis, as well as new proteins exhibiting such glial cell mitogenic activity. In

addition, DNA encoding these proteins and antibodies which bind these and related proteins are provided.

The novel proteins of the invention include alternative splicing products of sequences encoding known polypeptides. Generally, these known proteins are members of the GGF/p185<sup>erbB2</sup> family of proteins.

Specifically, the invention provides polypeptides of a specified formula, and DNA sequences encoding those polypeptides. The polypeptides have the formula

10

WYBAZCX

wherein WYBAZCX is composed of the amino acid sequences shown in Figure 31 (SEQ ID Nos. 136-139, 141-147, 160, 161, 173-178, 42-44, 77); wherein W comprises the polypeptide segment F, or is absent; wherein Y comprises the polypeptide segment E, or is absent; wherein Z comprises the polypeptide segment G or is absent; and wherein X comprises the polypeptide segments C/D HKL, C/D H, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, or C/D C/D' D' HKL; provided that, either

a) at least one of F, Y, B, A, Z, C, or X is of bovine origin; or

b) Y comprises the polypeptide segment E; or

25 c) X comprises the polypeptide segments C/D HKL, C/D D, C/D' HKL, C/D C/D' HKL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, C/D C/D' D' HKL, C/D'H, C/D C/D'H, or C/D C/D' HL.

In addition, the invention includes the DNA sequence comprising coding segments 'FBA' as well as the with

30

corresponding polypeptide segments having the amino acid sequences shown in Figure 31 (SEQ ID Nos. 136, 138, 139, 173-175);

5 the DNA sequence comprising the coding segments 'FBA'' as well as the corresponding polypeptide segments having the amino acid sequences shown in Figure 31 (SEQ ID Nos. 136, 138, 140, 173, 174);

the DNA sequence comprising the coding segments 'FEBA'' as well as the corresponding polypeptide segments  
10 having the amino acid sequences shown in Figure 31 (SEQ ID Nos. 136-139, 173-175);

the DNA sequence comprising the coding segments 'FEBA'' as well as the corresponding polypeptide segments having the amino acid sequences shown in Figure 31 (SEQ ID  
15 Nos. 136-138, 140, 173, 174); and

the DNA sequence comprising the polypeptide coding segments of the GGF2HBS5 cDNA clone (ATCC Deposit No. 75298, deposited September 2, 1992).

The invention further includes peptides of the  
20 formula FBA, FEBA, FBA' FEBA' and DNA sequences encoding these peptides wherein the polypeptide segments correspond to amino acid sequences shown in Figure 31, SEQ ID Nos. (136, 138, 139, 173-175), (136-139, 173-175) and (136, 138, 140, 173, 174) and (136-138, 140, 173, 174) respectively.  
25 The polypeptide purified GGF-II polypeptide (SEQ ID No. 167) is also included as a part of the invention.

Further included as an aspect of the invention are peptides and DNA encoding such peptides which are useful for the treatment of glia and in particular oligodendrocytes,  
30 microglia and astrocytes, of the central nervous system and methods for the administration of these peptides.



Also included in this invention is the mature GGF peptide and the DNA encoding said peptide, exclusive of the N-terminal signal sequence, which is also useful for the treatment of conditions of the central nervous system and for the preparation of antibodies specific for said peptides. These antibodies may be useful for purification of peptides described herein and for diagnostic applications.

The invention further includes vectors including DNA sequences which encode the amino acid sequences, as defined above. Also included are a host cell containing the isolated DNA encoding the amino acid sequences, as defined above. The invention further includes those compounds which bind the p185<sup>erbB2</sup> receptor and stimulate glial cell mitogenesis *in vivo* and/or *in vitro*.

Also a part of the invention are antibodies to the novel peptides described herein. In addition, antibodies to any of the peptides described herein may be used for the purification of polypeptides described herein. The antibodies to the polypeptides may also be used for the therapeutic inhibitor of glial cell mitogenesis.

The invention further provides a method for stimulating glial cell mitogenesis comprising contacting glial cells with a polypeptide defined by the formula

25

WYBAZCX

wherein WYBAZCX is composed of the polypeptide segments shown in Figure 31 (SEQ ID Nos. 136-139, 141-147, 160, 161, 173-178, 42-44, 77); wherein W comprises the polypeptide segment F, or is absent wherein Y comprises the polypeptide segment E, or is absent; wherein Z comprises the

30

polypeptide segment G or is absent; and wherein X comprises the polypeptide segment C/D HKL, C/D H, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, or C/D C/D' D' HKL.

The invention also includes a method for the preparation of a glial cell mitogenic factor which consists of culturing modified host cells as defined above under conditions permitting expression of the DNA sequences of the invention.

The peptides of the invention can be used to make a pharmaceutical or veterinary formulation for pharmaceutical or veterinary use. Optionally, the formulation may be used together with an acceptable diluent, carrier or excipient and/or in unit dosage form.

A method for stimulating mitogenesis of a glial cell by contacting the glial cell with a polypeptide defined above as a glial cell mitogen *in vivo* or *in vitro* is also an aspect of the invention. A method for producing a glial cell mitogenic effect in a vertebrate (preferably a mammal, more preferably a human) by administering an effective amount of a polypeptide as defined is also a component of the invention.

Methods for treatment of diseases and disorders using the polypeptides described are also a part of the invention. For instance, a method of treatment or prophylaxis for a nervous disease or disorder can be effected with the polypeptides described. Also included are a method for the prophylaxis or treatment of a pathophysiological condition of the nervous system in which

a cell type is involved which is sensitive or responsive to a polypeptide as defined are a part of the invention.

Included in the invention as well, are methods for treatment when the condition involves peripheral nerve  
5 damage; nerve damage in the central nervous system; neurodegenerative disorders; demyelination in peripheral or central nervous system; or damage or loss of Schwann cells oligodendrocytes, microglia, or astrocytes. For example a neuropathy of sensory or motor nerve fibers; or the  
10 treatment of a neurodegenerative disorder are included. In any of these cases, treatment consists of administering an effective amount of the polypeptide.

The invention also includes a method for inducing neural regeneration and/or repair by administering an  
15 effective amount of a polypeptide as defined above. Such a medicament is made by administering the polypeptide with a pharmaceutically effective carrier.

The invention includes the use of a polypeptide as defined above in the manufacture of a medicament.

20 The invention further includes the use of a polypeptide as defined above

-to immunize a mammal for producing antibodies, which can optionally be used for therapeutic or diagnostic purposes

25 -in a competitive assay to identify or quantify molecules having receptor binding characteristics corresponding to those of the polypeptide; and/or

-for contacting a sample with a polypeptide, as mentioned above, along with a receptor capable of binding  
30 specifically to the polypeptide for the purpose of detecting competitive inhibition of binding to the polypeptide.

-in an affinity isolation process, optionally affinity chromatography, for the separation of a corresponding receptor.

5 The invention also includes a method for the prophylaxis or treatment of a glial tumor. This method consists of administering an effective amount of a substance which inhibits the binding of a factor as defined by the peptides above.

10 Furthermore, the invention includes a method of stimulating glial cell mitogenic activity by the application to the glial cell of a

-30 kD polypeptide factor isolated from the MDA - MB 231 human breast cell line; or

15 -35 kD polypeptide factor isolated from the rat I-EJ transformed fibroblast cell line to the glial cell or

-75 kD polypeptide factor isolated from the SKBR-3 human breast cell line; or

-44 kD polypeptide factor isolated from the rat I-EJ transformed fibroblast cell line; or

20 -25kD polypeptide factor isolated from activated mouse peritoneal macrophages; or

-45 kD polypeptide factor isolated from the MDA - MB 231 human breast cell; or

25 -7 to 14 kD polypeptide factor isolated from the ATL-2 human T-cell line to the glial cell; or

-25 kD polypeptide factor isolated from the bovine kidney cell; or

-42 kD polypeptide factor (ARIA) isolated from brains.

30 The invention further includes a method for the use of the EGFL1, EGFL2, EGFL3, EGFL4, EGFL5, and EGFL6

polypeptides, Figure 38 to 43 and SEQ ID Nos. 154 to 159, respectively, for the stimulation of glial cell mitogenesis in vivo and in vitro.

Also included in the invention is the administration  
5 of the GGF-II polypeptide whose sequence is shown in Figure 45 for the stimulation of glial cell mitogenesis.

An additional aspect of the invention includes the use of the above-referenced peptides for the purpose of stimulating Schwann cells to produce growth factors which  
10 may, in turn, be harvested for scientific or therapeutic use.

Furthermore, the peptides described herein may be used to induce central glial proliferation and remyelination for treatment of diseases, e.g., MS, where re-myelination is  
15 desired.

In an additional aspect of the invention, the novel polypeptides described herein may be used to stimulate the synthesis of acetylcholine receptors.

As mentioned above, the invention provides new glial  
20 growth factors from mammalian sources, including bovine and human, which are distinguished from known factors. These factors are mitogenic for Schwann cells against a background of fetal calf plasma (FCP). The invention also provides processes for the preparation of these factors, and an  
25 improved method for defining activity of these and other factors. Therapeutic application of the factors is a further significant aspect of the invention.

Thus, important aspects of the invention are:

(a) a basic polypeptide factor having glial cell  
30 mitogenic activity, more specifically, Schwann cell mitogenic activity in the presence of fetal calf plasma, a

molecular weight of from about 30 kD to about 36 kD, and including within its amino acid sequence any one or more of the following peptide sequences:

5            F K G D A H T E  
            A S L A D E Y E Y M X K  
            T E T S S S G L X L K  
            A S L A D E Y E Y M R K  
            A G Y F A E X A R  
            T T E M A S E Q G A  
10            A K E A L A A L K  
            F V L Q A K K  
            E T Q P D P G Q I L K K V P M V I G A Y T  
            E Y K C L K F K W F K K A T V M  
            E X K F Y V P  
15            K L E F L X A K; and

(b) a basic polypeptide factor which stimulates glial cell mitogenesis, particularly the division of Schwann cells, in the presence of fetal calf plasma, has a molecular weight of from about 55 kD to about 63 kD, and including  
20 within its amino acid sequence any one or more of the following peptide sequences:

            V H Q V W A A K  
            Y I F F M E P E A X S S G  
            L G A W G P P A F P V X Y  
25            W F V V I E G K  
            A S P V S V G S V Q E L Q R  
            V C L L T V A A L P P T  
            K V H Q V W A A K

K A S L A D S G E Y M X K  
D L L L X V  
E G K V H P Q R R G A L D R K  
P S C G R L K E D S R Y I F F M E  
5 E L N R K N K P Q N I K I Q K K

The novel peptide sequences set out above, derived from the smaller molecular weight polypeptide factor, and from the larger molecular weight polypeptide factor, are also aspects of this invention in their own right. These  
10 sequences are useful as probe sources for polypeptide factors of the invention, for investigating, isolating or preparing such factors (or corresponding gene sequences) from a range of different species, or preparing such factors by recombinant technology, and in the generation of  
15 corresponding antibodies, by conventional technologies, preferably monoclonal antibodies, which are themselves useful investigative tools and are possible therapeutics. The invention also includes an isolated glial cell mitogenic activity encoding gene sequence, or fragment thereof,  
20 obtainable by the methods set out above for the novel peptide sequences of the invention.

The availability of short peptides from the highly purified factors of the invention has enabled additional sequences to be determined (see Examples to follow).

25 Thus, the invention further embraces a polypeptide factor having glial cell mitogenic activity and including an amino acid sequence encoded by:

(a) a DNA sequence shown in any one of Figures 28a, 28b or 28c, SEQ ID Nos. 133-135, respectively;

(b) a DNA sequence shown in Figure 22, SEQ ID No. 89;

(c) the DNA sequence represented by nucleotides 281-557 of the sequence shown in Figure 28a, SEQ ID No. 133;  
5 or

(d) a DNA sequence hybridizable to any one of the DNA sequences according to (a), (b) or (c).

The invention further includes sequences which have greater than 60%, preferably 80%, sequence identity of  
10 homology to the sequences indicated above.

While the present invention is not limited to a particular set of hybridization conditions, the following protocol gives general guidance which may, if desired, be followed:

15 DNA probes may be labelled to high specific activity (approximately  $10^8$  to  $10^{9.5}$  dpm/ $\mu$ g) by nick-translation or by PCR reactions according to Schowalter and Sommer (Anal. Biochem., 177:90-94, 1989) and purified by desalting on  
20 G-150 Sephadex columns. Probes may be denatured (10 minutes in boiling water followed by immersion into ice water), then added to hybridization solutions of 80% buffer B (2g polyvinylpyrrolidone, 2g Ficoll-400, 2g bovine serum albumin, 50ml 1 M Tris HCL (pH 7.5), 58g NaCl, 1g sodium pyrophosphate, 10g sodium dodecyl sulfate, 950ml H<sub>2</sub>O)  
25 containing 10% dextran sulfate at  $10^6$  dpm  $^{32}$ P per ml and incubated overnight (approximately 16 hours) at 60°C. The filters may then be washed at 60°C, first in buffer B for 15 minutes followed by three 20-minute washes in 2X SSC, 0.1% SDS then one for 20 minutes in 1X SSC, 0.1% SDS.

30 In other respects, the invention provides:



(a) a basic polypeptide factor which has, if obtained from bovine pituitary material, an observed molecular weight, whether in reducing conditions or not, of from about 30kD to about 36kD on SDS-polyacrylamide gel electrophoresis using the following molecular weight standards:

	Lysozyme (hen egg white)	14,400
	Soybean trypsin inhibitor	21,500
	Carbonic anhydrase (bovine)	31,000
10	Ovalbumin (hen egg white)	45,000
	Bovine serum albumin	66,200
	Phosphorylase B (rabbit muscle)	97,400;

which factor has glial cell mitogenic activity including stimulating the division of rat Schwann cells in the presence of fetal calf plasma, and when isolated using reversed-phase HPLC retains at least 50% of said activity after 10 weeks incubation in 0.1% trifluoroacetic acid at 4°C; and

(b) a basic polypeptide factor which has, if obtained from bovine pituitary material, an observed molecular weight, under non-reducing conditions, of from about 55 kD to about 63 kD on SDS-polyacrylamide gel electrophoresis using the following molecular weight standards:

25	Lysozyme (hen egg white)	14,400
	Soybean trypsin inhibitor	21,500
	Carbonic anhydrase (bovine)	31,000
	Ovalbumin (hen egg white)	45,000
	Bovine serum albumin	66,200

Phosphorylase B (rabbit muscle) 97,400;

which factor the human equivalent of which is encoded by DNA clone GGF2HBS5 described herein and which factor has glial cell mitogenic activity including stimulating the division  
5 of rat Schwann cells in the presence of fetal calf plasma, and when isolated using reversed-phase HPLC retains at least 50% of the activity after 4 days incubation in 0.1% trifluoroacetic acid at 4°C.

For convenience of description only, the lower  
10 molecular weight and higher molecular weight factors of this invention are referred to hereafter as "GGF-I" and "GGF-II", respectively. The "GGF2" designation is used for all clones isolated with peptide sequence data derived from GGF-II protein (i.e., GGF2HBS5, GGF2BPP3).

15 It will be appreciated that the molecular weight range limits quoted are not exact, but are subject to slight variations depending upon the source of the particular polypeptide factor. A variation of, say, about 10% would not, for example, be impossible for material from another  
20 source.

Another important aspect of the invention is a DNA sequence encoding a polypeptide having glial cell mitogenic activity and comprising:

(a) a DNA sequence shown in any one of Figures 28a,  
25 28b or 28c, SEQ ID Nos. 133-135:

(b) a DNA sequence shown in Figure 22, SEQ ID No. 89;

(c) the DNA sequence represented by nucleotides 281-557 of the sequence shown in Figure 28a, SEQ ID No. 133;  
30 or

(d) a DNA sequence hybridizable to any one of the DNA sequences according to (a), (b) or (c).

Another aspect of the present invention uses the fact that the Glial Growth Factors and p185<sup>erbB2</sup> ligand proteins are encoded by the same gene. A variety of messenger RNA splicing variants (and their resultant proteins) are derived from this gene and many of these products show p185<sup>erbB2</sup> binding and activation. Several of the (GGF-II) gene products have been used to show Schwann cell mitogenic activity. This invention provides a use for all of the known products of the GGF/p185<sup>erbB2</sup> ligand gene (described in the references listed above) as Schwann cell mitogens.

This invention also relates to other, not yet naturally isolated splicing variants of the Glial Growth Factor gene. Figure 30, shows the known patterns of splicing derived from polymerase chain reaction experiments (on reverse transcribed RNA) and analysis of cDNA clones (as presented within) and derived from what has been published as sequences encoding p185<sup>erbB2</sup> ligands (Peles et al., Cell 69:205 (1992) and Wen et al., Cell 69:559 (1992)). These patterns, as well as additional ones disclosed herein, represent probable splicing variants which exist. Thus another aspect of the present invention relates to the nucleotide sequences encoding novel protein factors derived from this gene. The invention also provides processes for the preparation of these factors. Therapeutic application of these new factors is a further aspect of the invention.

Thus other important aspects of the invention are :

(a) A series of human and bovine polypeptide factors having glial cell mitogenic activity including

stimulating the division of Schwann cells. These peptide sequences are shown in Figures 31, 32, 33 and 34, SEQ ID Nos. 136-137, 173, respectively.

(b) A series of polypeptide factors having glial cell mitogenic activity including stimulating the division of Schwann cells and purified and characterized according to the procedures outlined by Lupu et al. Science 249: 1552 (1990); Lupu et al. Proc. Natl. Acad. Sci USA 89: 2287 (1992); Holmes et al. Science 256: 1205 (1992); Peles et al. 69: 205 (1992); Yarden and Peles Biochemistry 30: 3543 (1991); Dobashi et al. Proc. Natl. Acad. Sci. 88: 8582 (1991); Davis et al. Biochem. Biophys. Res. Commun. 179: 1536 (1991); Beaumont et al., patent application PCT/US91/03443 (1990); Greene et al. patent application PCT/US91/02331 (1990); Usdin and Fischbach, J. Cell. Biol. 103:493-507 (1986); Falls et al., Cold Spring Harbor Symp. Quant. Biol. 55:397-406 (1990); Harris et al., Proc. Natl. Acad. Sci. USA 88:7664-7668 (1991); and Falls et al., Cell 72:801-815 (1993).

(c) A polypeptide factor (GGFBPP5) having glial cell mitogenic activity including stimulating the division of Schwann cells. The amino acid sequence is shown in Figure 32, SEQ ID No. 148, and is encoded by the bovine DNA sequence shown in Figure 32, SEQ ID No. 148.

The novel human peptide sequences described above and presented in Figures 31, 32, 33 and 34, SEQ ID Nos. 136-150, 173-176, 178, 42-44, 77, respectively, represent a series of splicing variants which can be isolated as full length complementary DNAs (cDNAs) from natural sources (cDNA libraries prepared from the appropriate tissues) or can be

assembled as DNA constructs with individual exons (e.g., derived as separate exons) by someone skilled in the art.

Other compounds in particular, peptides, which bind specifically to the p185<sup>erbB2</sup> receptor can also be used according to the invention as a glial cell mitogen. A candidate compound can be routinely screened for p185<sup>erbB2</sup> binding, and, if it binds, can then be screened for glial cell mitogenic activity using the methods described herein.

The invention includes any modifications or equivalents of the above polypeptide factors which do not exhibit a significantly reduced activity. For example, modifications in which amino acid content or sequence is altered without substantially adversely affecting activity are included. By way of illustration, in EP-A 109748 mutations of native proteins are disclosed in which the possibility of unwanted disulfide bonding is avoided by replacing any cysteine in the native sequence which is not necessary for biological activity with a neutral amino acid. The statements of effect and use contained herein are therefore to be construed accordingly, with such uses and effects employing modified or equivalent factors being part of the invention.

The new sequences of the invention open up the benefits of recombinant technology. The invention thus also includes the following aspects:

(a) DNA constructs comprising DNA sequences as defined above in operable reading frame position within vectors (positioned relative to control sequences so as to permit expression of the sequences) in chosen host cells after transformation thereof by the constructs (preferably the control sequence includes regulatable promoters, e.g.

Trp). It will be appreciated that the selection of a promoter and regulatory sequences (if any) are matters of choice for those of skill in the art;

5 (b) host cells modified by incorporating constructs as defined in (a) immediately above so that said DNA sequences may be expressed in said host cells - the choice of host is not critical, and chosen cells may be prokaryotic or eukaryotic and may be genetically modified to incorporate said constructs by methods known in the art; and,

10 (c) a process for the preparation of factors as defined above comprising cultivating the modified host cells under conditions permitting expression of the DNA sequences. These conditions can be readily determined, for any particular embodiment, by those of skill in the art of  
15 recombinant DNA technology. Glial cell mitogens prepared by this means are included in the present invention.

None of the factors described in the art has the combination of characteristics possessed by the present new polypeptide factors.

20 As indicated, the Schwann cell assay used to characterize the present factors employs a background of fetal calf plasma. In all other respects, the assay can be the same as that described by Brockes et al. in Meth. Enz., supra, but with 10% FCP replacing 10% FCS. This difference  
25 in assay techniques is significant, since the absence of platelet-derived factors in fetal calf plasma (as opposed to serum) enables a more rigorous definition of activity on Schwann cells by eliminating potentially spurious effects from some other factors.

30 The invention also includes a process for the preparation of a polypeptide as defined above, extracting

vertebrate brain material to obtain protein, subjecting the resulting extract to chromatographic purification by hydroxylapatite HPLC and then subjecting these fractions to SDS-polyacrylamide gel electrophoresis. The fraction which  
5 has an observed molecular weight of about 30kD to 36 kD and/or the fraction which has an observed molecular weight of about 55kD to 63 kD is collected. In either case, the fraction is subjected to SDS-polyacrylamide gel electrophoresis using the following molecular weight  
10 standards:

	Lysozyme (hen egg white)	14,400
	Soybean trypsin inhibitor	21,500
	Carbonic anhydrase (bovine)	31,000
	Ovalbumin (hen egg white)	45,000
15	Bovine serum albumin	66,200
	Phosphorylase B (rabbit muscle)	97,400

In the case of the smaller molecular weight fraction, the SDS-polyacrylamide gel is run in non-reducing conditions in reducing conditions or, and in the case of the larger  
20 molecular weight fraction the gel is run under non-reducing conditions. The fractions are then tested for activity stimulating the division of rat Schwann cells against a background of fetal calf plasma.

Preferably, the above process starts by isolating a  
25 relevant fraction obtained by carboxymethyl cellulose chromatography, e.g. from bovine pituitary material. It is also preferred that hydroxylapatite HPLC, cation exchange chromatography, gel filtration, and/or reversed-phase HPLC be employed prior to the SDS-Polyacrylamide gel  
30 electrophoresis. At each stage in the process, activity may be determined using Schwann cell incorporation of

radioactive iododeoxyuridine as a measure in an assay generally as described by Brockes in Meth. Enz., supra, but modified by substituting 10% FCP for 10% FCS. As already noted, such an assay is an aspect of the invention in its own substance for CNS or PNS cell, e.g. Schwann cell, mitogenic effects.

Thus, the invention also includes an assay for glial cell mitogenic activity in which a background of fetal calf plasma is employed against which to assess DNA synthesis in glial cells stimulated (if at all) by a substance under assay.

Another aspect of the invention is a pharmaceutical or veterinary formulation comprising any factor as defined above formulated for pharmaceutical or veterinary use, respectively, optionally together with an acceptable diluent, carrier or excipient and/or in unit dosage form. In using the factors of the invention, conventional pharmaceutical or veterinary practice may be employed to provide suitable formulations or compositions.

Thus, the formulations of this invention can be applied to parenteral administration, for example, intravenous, subcutaneous, intramuscular, intraorbital, ophthalmic, intraventricular, intracranial, intracapsular, intraspinal, intracisternal, intraperitoneal, topical, intranasal, aerosol, scarification, and also oral, buccal, rectal or vaginal administration.

The formulations of this invention may also be administered by the transplantation into the patient of host cells expressing the DNA of the instant invention or by the use of surgical implants which release the formulations of the invention.



Parenteral formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are to be found in, for example, "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain as excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes, biocompatible, biodegradable lactide polymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the present factors. Other potentially useful parenteral delivery systems for the factors include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain as excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel to be applied intranasally. Formulations for parenteral administration may also include glycocholate for buccal administration, methoxysalicylate for rectal administration, or citric acid for vaginal administration.

The present factors can be used as the sole active agents, or can be used in combination with other active ingredients, e.g., other growth factors which could

facilitate neuronal survival in neurological diseases, or peptidase or protease inhibitors.

The concentration of the present factors in the formulations of the invention will vary depending upon a number of issues, including the dosage to be administered, and the route of administration.

In general terms, the factors of this invention may be provided in an aqueous physiological buffer solution containing about 0.1 to 10% w/v compound for parenteral administration. General dose ranges are from about 1 mg/kg to about 1 g/kg of body weight per day; a preferred dose range is from about 0.01 mg/kg to 100 mg/kg of body weight per day. The preferred dosage to be administered is likely to depend upon the type and extent of progression of the pathophysiological condition being addressed, the overall health of the patient, the make up of the formulation, and the route of administration.

As indicated above, Schwann cells (the glial cells of the peripheral nervous system) are stimulated to divide in the presence of the factors of the invention. Schwann cells of the peripheral nervous system are involved in supporting neurons and in creating the myelin sheath around individual nerve fibers. This sheath is important for proper conduction of electrical impulses to muscles and from sensory receptors.

There are a variety of peripheral neuropathies in which Schwann cells and nerve fibers are damaged, either primarily or secondarily. There are many neuropathies of both sensory and motor fibers (Adams and Victor, Principles of Neurology). The most important of those neuropathies are probably the neuropathies associates with diabetes, multiple

sclerosis, Landry-Guillain-Barr syndrome, neuropathies caused by carcinomas, and neuropathies caused by toxic agents (some of which are used to treat carcinomas).

The invention, however, envisages treatment or  
5 prophylaxis of conditions where nervous system damage has been brought about by any basic cause, e.g. infection or injury. Thus, in addition to use of the present factors in the treatment of disorders or diseases of the nervous system where demyelination or loss of Schwann cells is present,  
10 such glial growth factors can be valuable in the treatment of disorders of the nervous system that have been caused by damage to the peripheral nerves. Following damage to peripheral nerves, the regeneration process is led by the growth or the re-establishment of Schwann cells, followed by  
15 the advancement of the nerve fibre back to its target. By speeding up the division of Schwann cells one could promote the regenerative process following damage.

Similar approaches could be used to treat injuries or neurodegenerative disease of the central nervous system  
20 (brain and spinal cord).

Furthermore, there are a variety of tumors of glial cells the most common of which is probably neurofibromatosis, which is a patchy small tumor created by overgrowth of glial cells. Also, it has been found that an  
25 activity very much like GGF can be found in some Schwann cell tumors, and therefore inhibitors of the action of the present factors on their receptors provides a therapy of a glial tumor, which comprises administering an effective amount of a substance which inhibits the binding of a  
30 factor, as defined above, to a receptor.

In general, the invention includes the use of present polypeptide factors in the prophylaxis or treatment of any pathophysiological condition of the nervous system in which a factor-sensitive or factor-responsive cell type is  
5 involved.

The polypeptide factors of the invention can also be used as immunogens for making antibodies, such as monoclonal antibodies, following standard techniques. Such antibodies are included within the present invention. These antibodies  
10 can, in turn, be used for therapeutic or diagnostic purposes. Thus, conditions perhaps associated with abnormal levels of the factor may be tracked by using such antibodies. In vitro techniques can be used, employing assays on isolated samples using standard methods. Imaging  
15 methods in which the antibodies are, for example, tagged with radioactive isotopes which can be imaged outside the body using techniques for the art of tumour imaging may also be employed.

The invention also includes the general use of the present factors as glial cell mitogens in vivo or in vitro, and the factors for such use. One specific embodiment is thus a method for producing a glial cell mitogenic effect in a vertebrate by administering an effective amount of a factor of the invention. A preferred embodiment is such a  
20 method in the treatment or prophylaxis of a nervous system disease or disorder.

A further general aspect of the invention is the use of a factor of the invention in the manufacture of a medicament, preferably for the treatment of a nervous  
25 disease or disorder, or for neural regeneration or repair.

Also included in the invention are the use of the factors of the invention in competitive assays to identify or quantify molecules having receptor binding characteristics corresponding to those of said polypeptides.

- 5 The polypeptides may be labelled, optionally with a radioisotope. A competitive assay can identify both antagonists and agonists of the relevant receptor.

In another aspect, the invention provides the use of each one of the factors of the invention in an affinity  
10 isolation process, optionally affinity chromatography, for the separation of a respective corresponding receptor. Such processes for the isolation of receptors corresponding to particular proteins are known in the art, and a number of techniques are available and can be applied to the factors  
15 of the present invention. For example, in relation to IL-6 and IFN $\gamma$  the reader is referred to Novick, D.; et al., J. Chromatogr. (1990) 510: 331-7. With respect to gonadotropin releasing hormone reference is made to Hazum, E., J. (1990) Chromatogr. 510:233-8. In relation to G-CSF reference is  
20 made to Fukunaga, R., et al., J. Biol. Chem., 265:13386-90. In relation to IL-2 reference is made to Smart, J.E., et al., (1990) J. Invest. Dermatol., 94:158S-163S, and in relation to human IFN-gamma reference is made to Stefanos, S, et al., (1989) J. Interferon Res., 9:719-30.

25 Brief Description of the Drawings

The drawings will first be described.

Drawings

Figures 1 to 8 relate to Example 1, and are briefly described below:

Fig. 1 is the profile for product from carboxymethyl cellulose chromatography;

Fig. 2 is the profile for product from hydroxylapatite HPLC;

5 Fig. 3 is the profile for product from Mono S FPLC;

Fig. 4 is the profile for product from Gel filtration FPLC;

10 Figs. 5 and 6 depict the profiles for the two partially purified polypeptide products from reversed-phase HPLC; and

Figs. 7 and 8 depict dose-response curves for the GGF-I and GGF-II fractions from reversed-phase HPLC using either a fetal calf serum or a fetal calf plasma background;

15 Figs. 9 to 12 depict the peptide sequences derived from GGF-I and GGF-II, SEQ ID Nos. 1-20, 22-29, 32-53 and 169, (see Example 2 hereinafter), Figures 10 and 12 specifically depict novel sequences:

In Fig. 10, Panel A, the sequences of GGF-I peptides used to design degenerate oligonucleotide probes and  
20 degenerate PCR primers are listed (SEQ ID Nos. 20, 1, 22-29, and 17). Some of the sequences in Panel A were also used to design synthetic peptides. Panel B is a listing of the sequences of novel peptides that were too short (less than 6 amino acids) for the design of degenerate probes or  
25 degenerate PCR primers (SEQ ID Nos. 17 and 52);

In Fig. 12, Panel A, is a listing of the sequences of GGF-II peptides used to design degenerate oligonucleotide probes and degenerate PCR primers (SEQ ID Nos. 45-52). Some of the sequences in Panel A were used to design synthetic  
30 peptides. Panel B is a listing of the novel peptide that

was too short (less than 6 amino acids) for the design of degenerate probes or degenerate PCR primers (SEQ ID No. 53);

Figures 13 to 20 relate to Example 3, below and depict the mitogenic activity of factors of the invention;

5       Figures 21 to 28 (a, b and c) relate to Example 4, below and are briefly described below:

Fig. 21 is a listing of the degenerate oligonucleotide probes (SEQ ID Nos. 54-88) designed from the novel peptide sequences in Figure 10, Panel A and Figure 12,  
10   Panel A;

Fig. 22 (SEQ ID No. 89) depicts a stretch of the putative bovine GGF-II gene sequence from the recombinant bovine genomic phage GGF2BG1, containing the binding site of degenerate oligonucleotide probes 609 and 650 (see Figure  
15   21, SEQ ID NOS. 69 and 72, respectively). The figure is the coding strand of the DNA sequence and the deduced amino acid sequence in the third reading frame. The sequence of peptide 12 from factor 2 (bold) is part of a 66 amino acid open reading frame (nucleotides 75272);

20       Fig. 23 is the degenerate PCR primers (Panel A, SEQ IS Nos. 90-108) and unique PCR primers (Panel B, SEQ ID Nos. 109-119) used in experiments to isolate segments of the bovine GGF-II coding sequences present in RNA from posterior pituitary;

25       Fig. 24 depicts of the nine distinct contiguous bovine GGF-II cDNA structures and sequences that were obtained in PCR amplification experiments using the list of primers in Figure 7, Panels A and B, and RNA from posterior pituitary. The top line of the Figure is a schematic of the  
30   coding sequences which contribute to the cDNA structures that were characterized;

Fig. 25 is a physical map of bovine recombinant phage of GGF2BG1. The bovine fragment is roughly 20 kb in length and contains two exons (bold) of the bovine GGF-II gene. Restriction sites for the enzymes XbaI, SpeI, NdeI, EcoRI, KpnI, and SstI have been placed on this physical map. Shaded portions correspond to fragments which were subcloned for sequencing;

Fig. 26 is a schematic of the structure of three alternative gene products of the putative bovine GGF-II gene. Exons are listed A through E in the order of their discovery. The alternative splicing patterns 1, 2 and 3 generate three overlapping deduced protein structures (GGF2BPP1, 2, and 3), which are displayed in the various Figures 28a, b, c (described below);

Fig. 27 (SEQ ID Nos. 120-132) is a comparison of the GGF-I and GGF-II sequences identified in the deduced protein sequences shown in Figures 28a, 28b and 28c (described below) with the novel peptide sequences listed in Figures 10 and 12. The Figure shows that six of the nine novel GGF-II peptide sequences are accounted for in these deduced protein sequences. Two peptide sequences similar to GGF-I sequences are also found;

Fig. 28a (SEQ ID No. 133) is a listing of the coding strand DNA sequence and deduced amino acid sequence of the cDNA obtained from splicing pattern number 1 in Figure 26. This partial cDNA of the putative bovine GGF-II gene encodes a protein of 206 amino acids in length. Peptides in bold were those identified from the lists presented in Figures 10 and 12. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA);



Fig. 28b (SEQ ID No. 134) is a listing of the coding strand DNA sequence and deduced amino acid sequence of the cDNA obtained from splicing pattern number 2 in Figure 26. This partial cDNA of the putative bovine GGF-II gene encodes a protein of 281 amino acids in length. Peptides in bold are those identified from the lists presented in Figures 10 and 12. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA);

Fig. 28c (SEQ ID No. 135) is a listing of the coding strand DNA sequence and deduced amino acid sequence of the cDNA obtained from splicing pattern number 3 in Figure 26. This partial cDNA of the putative bovine GGF-II gene encodes a protein of 257 amino acids in length. Peptides in bold are those identified from the lists in Figures 10 and 12. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA).

Fig. 29, which relates to Example 6 hereinafter, is an autoradiogram of a cross hybridization analysis of putative bovine GGF-II gene sequences to a variety of mammalian DNAs on a southern blot. The filter contains lanes of EcoRI-digested DNA (5  $\mu$ g per lane) from the species listed in the Figure. The probe detects a single strong band in each DNA sample, including a four kilobase fragment in the bovine DNA as anticipated by the physical map in Figure 25. Bands of relatively minor intensity are observed as well, which could represent related DNA sequences. The strong hybridizing band from each of the other mammalian DNA samples presumably represents the GGF-II homologue of those species.

Fig. 30 is a diagram of representative splicing variants. The coding segments are represented by F, E, B,

A, G, C, C/D, C/D', D, D', H, K and L. The location of the peptide sequences derived from purified protein are indicated by "o".

Fig. 31 (SEQ ID Nos. 136-147, 160, 161, 173-178, 42-  
5 44, 77) is a listing of the DNA sequences and predicted peptide sequences of the coding segments of GGF. Line 1 is a listing of the predicted amino acid sequences of bovine GGF, line 2 is a listing of the nucleotide sequences of bovine GGF, line 3 is a listing of the nucleotide sequences  
10 of human GGF (heregulin) (nucleotide base matches are indicated with a vertical line) and line 4 is a listing of the predicted amino acid sequences of human GGF/hereregulin where it differs from the predicted bovine sequence. Coding  
15 segments E, A' and K represent only the bovine sequences. Coding segment D' represents only the human (heregulin) sequence.

Fig. 32 (SEQ ID No. 148) is the predicted GGF2 amino acid sequence and nucleotide sequence of BPP5. The upper  
20 line is the nucleotide sequence and the lower line is the predicted amino acid sequence.

Fig. 33 (SEQ ID No. 149) is the predicted amino acid sequence and nucleotide sequence of GGF2BPP2. The upper  
line is the nucleotide sequence and the lower line is the predicted amino acid sequence.

25 Fig. 34 (SEQ ID No. 150) is the predicted amino acid sequence and nucleotide sequence of GGF2BPP4. The upper line is the nucleotide sequence and the lower line is the predicted amino acid sequence.

Fig. 35 (SEQ ID Nos. 151-152) depicts the alignment  
30 of two GGF peptide sequences (GGF2bpp4 and GGF2bpp5) with

the human EGF (hEGF). Asterisks indicate positions of conserved cysteines.

Fig. 36 depicts the level of GGF activity (Schwann cell mitogenic assay) and tyrosine phosphorylation of a ca. 200kD protein (intensity of a 200 kD band on an autoradiogram of a Western blot developed with an antiphosphotyrosine polyclonal antibody) in response to increasing amounts of GGF.

Fig. 37 is a list of splicing variants derived from the sequences shown in Figure 31.

Fig. 38 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL1 (SEQ ID No. 154).

Fig. 39 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL2 (SEQ ID No. 155).

Fig. 40 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL3 (SEQ ID No. 156).

Fig. 41 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL4 (SEQ ID No. 157).

Fig. 42 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL5 (SEQ ID No. 158).

Fig. 43 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL6 (SEQ ID No. 159).

Fig. 44 is a scale coding segment map of the clone. T3 refers to the bacteriophage promoter used to produce mRNA from the clone. R = flanking EcoRI restriction enzyme

5 sites. 5' UT refers to the 5' untranslated region. E, B, A, C, C/D', and D refer to the coding segments. O = the translation start site. A = the 5' limit of the region homologous to the bovine E segment (see example 6) and 3' UT refers to the 3' untranslated region.

10 Fig. 45 is the predicted amino acid sequence (middle) and nucleic sequence (top) of GGF2HBS5 (SEQ ID No. 167). The bottom (intermittent) sequence represents peptide sequences derived from GGF-II preparations (see Figures 11, 12).

Fig. 46 is a graph depicting the Schwann cell mitogenic activity of recombinant human and bovine glial growth factors.

15 Fig. 47 is a dose-response curve depicting Schwann cell proliferation activity data resulting from administration of different size aliquots of CHO cell conditioned medium.

20 Fig. 48 is a dose-response curve depicting Schwann cell mitogenic activity secreted into the extracellular medium by SF9 insect cells infected with baculovirus containing the GGF2HBS5 cDNA clone.

Fig. 49 is a Western blot of recombinant CHO cell conditioned medium using a GGF peptide antibody.

25 Fig. 50 (A) is a graph of Schwann cell proliferation activity of recombinant (COS cell produced) human GGF-II (rhGGF-II) peak eluted from the cation exchange column; (B) is an immunoblot against recombinant GGFII peak using polyclonal antibody made against specific peptide of rhGGFII;

30 Fig. 51 (A) is a graph showing the purification of rhGGF-II (CHO cell produced) on cation exchange column by

fraction; (B) is a photograph of a Western blot using fractions as depicted in (A) and a rhGGF-II specific antibody.

Fig. 52 is a photograph of a gel depicting tyrosine phosphorylation in Schwann cells treated with recombinant glial growth factors.

Fig. 53 is the sequences of GGFHBS5, GGFHFB1 and GGFBPP5 polypeptides (SEQ ID NOS: 170, 171, and 172).

Fig. 54 is a map of the CHO cell-expression vector pcDHFRpolyA.

Fig. 55 is the amino acid sequence of cDNA encoding mature hGGF2 (SEQ ID NO: 179).

#### Detailed Description

The invention pertains to the isolation and purification of novel Glial Growth factors and the cloning of DNA sequences encoding these factors. Other components of the invention are several gene splicing variants which potentially encode a series of glial growth factors, in particular the GGF2HBS5 in particular a variant which encodes the human equivalent of bovine GGF-II. It is evident that the gene encoding GGF's and p185<sup>erbB</sup> binding proteins produces a number of variably-sized, differentially-spliced RNA transcripts that give rise to a series of proteins, which are of different lengths and contain some common peptide sequences and some unique peptide sequences. This is supported by the differentially-spliced sequences which are recoverable from bovine posterior pituitary RNA (as presented herein), human breast cancer (MDA-MB-231) (Holmes et al. Science 256: 1205 (1992) and chicken brain RNA (Falls et al. Cell 72:1-20 (1993)).

Further support derives from the wide size range of proteins which act as both mitogens for Schwann cells (as disclosed herein) and as ligands for the p185<sup>erbB2</sup> receptor (see below).

Further evidence to support the fact that the genes  
5 encoding GGF and p185<sup>erbB2</sup> are homologous comes from nucleotide sequence comparison. Science, 256 (1992), 1205-1210) Holmes et al. demonstrate the purification of a 45-kilodalton human protein (Heregulin- $\alpha$ ) which specifically interacts with the receptor protein p185<sup>erbB2</sup>, which is  
10 associated with several human malignancies. Several complementary DNA clones encoding Heregulin- $\alpha$  were isolated. Peles et al. (Cell 69:205 (1992)) and Wen et al. (Cell 69:559 (1992)) describe a complementary DNA isolated from rat cells encoding a protein called "neu differentiation factor"  
15 (NDF). The translation product of the NDF cDNA has p185<sup>erbB2</sup> binding activity. Usdin and Fischbach, J. Cell. Biol. 103:493-507 (1986); Falls et al., Cold Spring Harbor Symp. Quant. Biol. 55:397-406 (1990); Harris et al., Proc. Natl. Acad. Sci. USA 88:7664-7668 (1991); and Falls et al., Cell  
20 72:801-815 (1993) demonstrate the purification of a 42 Kd glycoprotein which interacts with a receptor protein p185<sup>erbB2</sup> and several complementary cDNA clones were isolated (Falls et al. Cell 72:801-815 (1993). Several other groups have reported the purification of proteins of various molecular  
25 weights with p185<sup>erbB2</sup> binding activity. These groups include Lupu et al. (1992) Proc. Natl. Acad. Sci. USA 89:2287; Yarden and Peles (1991) Biochemistry 30:3543; Lupu et al. (1990) Science 249:1552; Dobashi et al. (1991) Biochem. Biophys. Res. Comm. 179:1536; and Huang et al. (1992) J.  
30 Biol. Chem. 257:11508-11512.

Other Embodiments

The invention includes any protein which is substantially homologous to the coding segments in Figure 31 (SEQ ID Nos. 136-147, 160, 161, 173-178, 42-44, 77) as well as other naturally occurring GGF polypeptides. Also  
5 included are: allelic variations; natural mutants; induced mutants; proteins encoded by DNA that hybridizes under high or low stringency conditions to a nucleic acid naturally occurring (for definitions of high and low stringency see  
10 Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1989, 6.3.1 - 6.3.6, hereby incorporated by reference); and polypeptides or proteins specifically bound by antisera to GGF polypeptide. The term also includes chimeric polypeptides that include the GGF polypeptides comprising sequences from Figure 31.

15 The following examples are not intended to limit the invention, but are provided to usefully illustrate the same, and provide specific guidance for effective preparative techniques.

As will be seen from Example 3, below, the present  
20 factors exhibit mitogenic activity on a range of cell types. The activity in relation to fibroblasts indicates a wound repair ability, and the invention encompasses this use. The general statements of invention above in relation to formulations and/or medicaments and their manufacture should  
25 clearly be construed to include appropriate products and uses. This is clearly a reasonable expectation for the present invention, given reports of similar activities for fibroblast growth factors (FGFs). Reference can be made, for example, to Sporn *et al.*, "Peptide Growth Factors and  
30 their Receptors I", page 396 (Baird and Bohlen) in the section headed "FGFs in Wound Healing and Tissue Repair".

EXAMPLE 1Purification of GGF-I and GGF-II from bovine PituitariesI. Preparation of Factor-CM Fraction

4,000 frozen whole bovine pituitaries (c.a. 12 kg)  
5 were thawed overnight, washed briefly with water and then  
homogenized in an equal volume of 0.15 M ammonium sulphate  
in batches in a Waring Blender. The homogenate was taken to  
pH 4.5 with 1.0 M HCl and centrifuged at 4,900g for 80  
minutes. Any fatty material in the supernatant was removed  
10 by passing it through glass wool. After taking the pH of the  
supernatant to 6.5 using 1.0 M NaOH, solid ammonium sulphate  
was added to give a 36% saturated solution. After several  
hours stirring, the suspension was centrifuged at 4,900 g  
for 80 minutes and the precipitate discarded. After  
15 filtration through glass wool, further solid ammonium  
sulphate was added to the supernatant to give a 75%  
saturated solution which was once again centrifuged at 4,900  
g for 80 minutes after several hours stirring. The pellet  
was resuspended in c.a. 2 L of 0.1 M sodium phosphate pH 6.0  
20 and dialyzed against 3 x 40 L of the same buffer. After  
confirming that the conductivity of the dialysate was below  
20.0  $\mu$ Siemens, it was loaded onto a Bioprocess column (120 x  
113 mm, Pharmacia) packed with carboxymethyl cellulose  
(CM-52, Whatman) at a flow rate of 2 ml min<sup>-1</sup>. The column  
25 was washed with 2 volumes of 0.1 M sodium phosphate pH 6.0,  
followed by 2 volumes of 50 mM NaCl, and finally 2 volumes  
of 0.2 M NaCl both in the same buffer. During the final  
step, 10 mL (5 minute) fractions were collected. Fractions  
73 to 118 inclusive were pooled, dialyzed against 10 volumes  
30 of 10 mM sodium phosphate pH 6.0 twice and clarified by  
centrifugation at 100,000 g for 60 minutes.



## II. Hydroxylapatite HPLC

Hydroxylapatite HPLC is not a technique hitherto used in isolating glial growth factors, but proved particularly efficacious in this invention.

- 5 The material obtained from the above CM-cellulose chromatography was filtered through a 0.22  $\mu$ m filter (Nalgene), loaded at room temperature on to a high performance hydroxylapatite column (50 x 50 mm, Biorad) equipped with a guard column (15 x 25 mm, Biorad) and
- 10 equilibrated with 10 mM potassium phosphate pH 6.0. Elution at room temperature was carried out at a flow rate of 2 ml.minute<sup>-1</sup> using the following programmed linear gradient:

	time (min)	%B	Solvent A: 10 mM potassium phosphate pH 6.0
	0.0	0	Solvent B: 1.0 M potassium phosphate pH 6.0
15	5.0	0	
	7.0	20	
	70.0	20	
	150.0	100	
	180.0	100	
20	185.0	0	

6.0 mL (3 minutes) fractions were collected during the gradient elution. Fractions 39-45 were pooled and dialyzed against 10 volumes of 50 mM sodium phosphate pH 6.0.

## III. Mono S FPLC

- 25 Mono S FPLC enabled a more concentrated material to be prepared for subsequent gel filtration.

Any particulate material in the pooled material from the hydroxylapatite column was removed by a clarifying spin at 100,000 g for 60 minutes prior to loading on to a

- preparative HR10/10 Mono S cation exchange column (100 x 10 mm, Pharmacia) which was then re-equilibrated to 50mM sodium phosphate pH 6.0 at room temperature with a flow rate of 1.0 ml/minute<sup>-1</sup>. Under these conditions, bound protein was
- 5 eluted using the following programmed linear gradient:

time (min)	%B	Solvent A: 50 mM potassium phosphate pH 6.0
0.0	0	Solvent B: 1.2 M sodium chloride, 50 mM
70.0	30	sodium phosphate pH 6.0
240.0	100	
10 250.0	100	
260.0	0	

1 mL (1 minute) fractions were collected throughout this gradient program. Fractions 99 to 115 inclusive were pooled.

15 IV. Gel Filtration FPLC

This step commenced the separation of the two factors of the invention prior to final purification, producing enriched fractions.

- For the purposes of this step, a preparative
- 20 Superose 12 FPLC column (510 x 20 mm, Pharmacia) was packed according to the manufacturers' instructions. In order to standardize this column, a theoretical plates measurement was made according to the manufacturers' instructions, giving a value of 9,700 theoretical plates.

- 25 The pool of Mono S eluted material was applied at room temperature in 2.5 Ml aliquots to this column in 50mM sodium phosphate, 0.75 NaCl pH 6.0 (previously passed through a C18 reversed phase column (Sep-pak, Millipore) at

a flow rate of 1.0 mL/minute<sup>1</sup>. 1 mL (0.5 minute) fractions were collected from 35 minutes after each sample was applied to the column. Fractions 27 to 41 (GGF-II) and 42 to 57 (GGF-I) inclusive from each run were pooled.

5 V. Reversed-Phase HPLC

The GGF-I and GGF-II pools from the above Superose 12 runs were each divided into three equal aliquots. Each aliquot was loaded on to a C8 reversed-phase column (Aquapore RP-300 7  $\mu$  C8 220 x 4.6 mm, Applied Biosystems) protected by a guard cartridge (RP-8, 15 x 3.2 mm, Applied Biosystems) and equilibrated to 40°C at 0.5 mL/minute. Protein was eluted under these conditions using the following programmed linear gradient:

time (min)	%B	Solvent A: 0.1% trifluoroacetic acid (TFA)
15 0		Solvent B: 90% acetonitrile, 0.1% TFA
60	66.6	
62.0	100	
72.0	100	
75.0	0	

20 200  $\mu$ L (0.4 minute) fractions were collected in siliconized tubes (Multilube tubes, Bioquote) from 15.2 minutes after the beginning of the programmed gradient.

VI. SDS-Polyacrylamide Gel Electrophoresis

In this step, protein molecular weight standards, low range, catalogue no. 161-0304, from Bio-Rad Laboratories Limited, Watford, England were employed. The actual proteins used, and their molecular weight standards, have been listed herein previously.

Fractions 47 to 53 (GGF-I) and fractions 61 to 67 (GGFII) inclusive from the reversed-phase runs were individually pooled. 7  $\mu$ L of the pooled material was boiled in an equal volume of 0.0125 M Tris-Cl, 4% SDS, 20% glycerol, and 10%  $\beta$ -mercaptoethanol for GGF-I, for 5 minutes and loaded on to an 11% polyacrylamide Laemmli gel with a 4% stacking gel and run at a constant voltage of 50 V for 16 hours. This gel was then fixed and stained using a silver staining kit (Amersham). Under these conditions, the factors are each seen as a somewhat diffuse band at relative molecular weights 30,000 to 36,000 Daltons (GGF-I) and 55,000 to 63,000 Daltons (GGFII) as defined by molecular weight markers. From the gel staining, it is apparent that there are a small number of other protein species present at equivalent levels to the GGF-I and GGF-II species in the material pooled from the reversed-phase runs.

#### VII. Stability in Trifluoroacetic Acid

Stability data were obtained for the present Factors in the presence of trifluoroacetic acid, as follows:-

20 GGF-I: Material from the reversed-phase HPLC, in the presence of 0.1% TFA and acetonitrile, was assayed within 12 hours of the completion of the column run and then after 10 weeks incubation at 40°C. Following incubation, the GGF-I had at least 50% of the activity of that material  
25 assayed directly off the column.

GGF-II: Material from the reversed-phase HPLC, in the presence of 0.1% TFA and acetonitrile, and stored at -20°C, was assayed after thawing and then after 4 days incubation at 40°C. Following incubation, the GGF-II had at  
30 least 50% of the activity of that material freshly thawed.

It will be appreciated that the trifluoroacetic acid concentration used in the above studies is that most commonly used for reversed-phase chromatography.

#### VIII. Activity Assay Conditions

5 Unless otherwise indicated, all operations were conducted at 37°C, and, with reference to Figures 1 to 6, activity at each stage was determined using the Brockes (Meth. Enz., supra) techniques with the following modifications. Thus, in preparing Schwann cells, 5 µM  
10 forskolin was added in addition to DMEM (Dulbecco's modified Eagle's medium), FCS and GGF. Cells used in the assay were fibroblast-free Schwann cells at passage number less than 10, and these cells were removed from flasks with trypsin and plated into flat-bottomed 96-well plates at 3.3 thousand  
15 cells per microwell.

[<sup>125</sup>I]IUdR was added for the final 24 hours after the test solution addition. The background (unstimulated) incorporation to each assay was less than 100 cpm, and maximal incorporation was 20 to 200 fold over background  
20 depending on Schwann cell batch and passage number.

In the case of the GGF-I and GGF-II fractions from reversed-phase HPLC as described above, two dose response curves were also produced for each factor, using exactly the above method for one of the curves for each factor, and the  
25 above method modified in the assay procedure only by substituting foetal calf plasma for fetal calf serum to obtain the other curve for each factor. The results are in Figures 7 and 8.

EXAMPLE 2Amino acid sequences of purified GGF-I and GGF-II

Amino acid sequence analysis studies were performed using highly purified bovine pituitary GGF-I and GGF-II.

5 The conventional single letter code was used to describe the sequences. Peptides were obtained by lysyl endopeptidase and protease V8 digests, carried out on reduced and carboxymethylated samples, with the lysyl endopeptidase digest of GGF-II carried out on material eluted from the  
10 55-65 RD region of a 11% SDS-PAGE (MW relative to the above-quoted markers).

A total of 21 peptide sequences (see Figure 9, SEQ ID Nos. 1-20, 169) were obtained for GGF-I, of which 12 peptides (see Figure 10, SEQ ID Nos. 1, 22-29, 17, 19, and  
15 32) are not present in current protein databases and therefore represent unique sequences. A total of 12 peptide sequences (see Figure 11, SEQ ID Nos. 33-39, 51, 52, 164-166) were obtained for GGF-II, of which 10 peptides (see Figure 12, SEQ ID Nos. 45-53) are not present in current  
20 protein databases and therefore represent unique sequences (an exception is peptide GGF-II 06 which shows identical sequences in many proteins which are probably of no significance given the small number of residues). These novel sequences are extremely likely to correspond to  
25 portions of the true amino acid sequences of GGFs I and II.

Particular attention can be drawn to the sequences of GGF-I 07 and GGF-II 12, which are clearly highly related. The similarities indicate that the sequences of these peptides are almost certainly those of the assigned GGF

species, and are most unlikely to be derived from contaminant proteins.

In addition, in peptide GGF-II 02, the sequence X S S is consistent with the presence of an N linked carbohydrate moiety on an asparagine at the position denoted by X.

In general, in Figures 9 and 11, X represents an unknown residue denoting a sequencing cycle where a single position could not be called with certainty either because there was more than one signal of equal size in the cycle or because no signal was present. An asterisk denotes those peptides where the last amino acid called corresponds to the last amino acid present in that peptide. In the remaining peptides, the signal strength after the last amino acid called was insufficient to continue sequence calling to the end of that peptide. The right hand column indicates the results of a computer database search using the GCG package FASTA and TFASTA programs to analyze the NBRF and EMBL sequence databases. The name of a protein in this column denotes identity of a portion of its sequence with the peptide amino acid sequence called allowing a maximum of two mismatches. A question mark denotes three mismatches allowed. The abbreviations used are as follows:

HMG-1	High Mobility Group protein-1
25 HMG-2	High Mobility Group protein-2
LH-alpha	Luteinizing hormone alpha subunit
LH-beta	Luteinizing hormone beta subunit

EXAMPLE 3Mitogenic Activity of Purified GGF-I and GGF-II

The mitogenic activity of a highly purified sample containing both GGFs I and II was studied using a quantitative method, which allows a single microculture to be examined for DNA synthesis, cell morphology, cell number and expression of cell antigens. This technique has been modified from a method previously reported by Muir et al., Analytical Biochemistry 185, 377-382, 1990. The main modifications are: 1) the use of uncoated microtiter plates, 2) the cell number per well, 3) the use of 5% Foetal Bovine Plasma (FBP) instead of 10% Foetal Calf Serum (FCS), and 4) the time of incubation in presence of mitogens and bromodeoxyuridine (BrdU), added simultaneously to the cultures. In addition the cell monolayer was not washed before fixation to avoid loss of cells, and the incubation time of monoclonal mouse anti-BrdU antibody and peroxidase conjugated goat anti-mouse immunoglobulin (IgG) antibody were doubled to increase the sensitivity of the assay. The assay, optimized for rat sciatic nerve Schwann cells, has also been used for several cell lines, after appropriate modifications to the cell culture conditions.

I. Methods of Mitogenesis Testing

On day 1, purified Schwann cells were plated onto uncoated 96 well plates in 5% FBP/Dulbecco's Modified Eagle Medium (DMEM) (5,000 cells/well). On day 2, GGFs or other test factors were added to the cultures, as well as BrdU at a final concentration of 10  $\mu$ m. After 48 hours (day 4) BrdU incorporation was terminated by aspirating the medium and



cells were fixed with 200  $\mu$ l/well of 70% ethanol for 20 min at room temperature. Next, the cells were washed with water and the DNA denatured by incubation with 100  $\mu$ l 2N HCl for 10 min at 37°C. Following aspiration, residual acid was

5 neutralized by filling the wells with 0.1 M borate buffer, pH 9.0, and the cells were washed with phosphate buffered saline (PBS). Cells were then treated with 50  $\mu$ l of blocking buffer (PBS containing 0.1% Triton X 100 and 2% normal goat serum) for 15 min at 37°C. After aspiration,

10 monoclonal mouse anti-BrdU antibody (Dako Corp., Santa Barbara, CA) (50  $\mu$ l/well, 1.4  $\mu$ g/ml diluted in blocking buffer) was added and incubated for two hours at 37°C. Unbound antibodies were removed by three washes in PBS containing 0.1% Triton X-100 and peroxidase-conjugated goat

15 anti-mouse IgG antibody (Dako Corp., Santa Barbara, CA) (50  $\mu$ l/well, 2  $\mu$ g/ml diluted in blocking buffer) was added and incubated for one hour at 37°C. After three washes in PBS/Triton and a final rinse in PBS, wells received 100  $\mu$ l/well of 50 mM phosphate/citrate buffer, pH 5.0,

20 containing 0.05% of the soluble chromogen o-phenylenediamine (OPD) and 0.02%  $H_2O_2$ . The reaction was terminated after 5-20 min at room temperature, by pipetting 80  $\mu$ l from each well to a clean plate containing 40  $\mu$ l/well of 2N sulfuric acid. The absorbance was recorded at 490nm using a plate reader

25 (Dynatech Labs). The assay plates containing the cell monolayers were washed twice with PBS and immunocytochemically stained for BrdU-DNA by adding 100  $\mu$ l/well of the substrate diaminobenzidine (DAB) and 0.02%  $H_2O_2$  to generate an insoluble product. After 10-20 min the

30 staining reaction was stopped by washing with water, and BrdU4-positive nuclei observed and counted using an inverted

microscope. occasionally, negative nuclei were counterstained with 0.001% Toluidine blue and counted as before.

## II. Cell lines used for Mitogenesis Assays

- 5        **Swiss 3T3 Fibroblasts:** Cells, from Flow Labs, were maintained in DMEM supplemented with 10% FCS, penicillin and streptomycin, at 37°C in a humidified atmosphere of 10% CO<sub>2</sub> in air. Cells were fed or subcultured every two days. For mitogenic assay, cells were plated at a density of 5,000
- 10 cells/well in complete medium and incubated for a week until cells were confluent and quiescent. The serum containing medium was removed and the cell monolayer washed twice with serum free-medium. 100 µl of serum free medium containing mitogens and 10µM of BrdU were added to each well and
- 15 incubated for 48 hours. Dose responses to GGFs and serum or PDGF (as a positive control) were performed.

- BHK (Baby Hamster Kidney) 21 C13 Fibroblasts:** Cells from European Collection of Animal Cell Cultures (ECACC), were maintained in Glasgow Modified Eagle Medium (GMEM)
- 20 supplemented with 5% tryptose phosphate broth, 5% FCS, penicillin and streptomycin, at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. Cells were fed or subcultured every two to three days. For mitogenic assay, cells were plated at a density of 2,000 cell/well in complete medium
- 25 for 24 hours. The serum containing medium was then removed and after washing with serum free medium, replaced with 100 µl of 0.1% FCS containing GMEM or GMEM alone. GGFs and FCS or bFGF as positive controls were added, coincident with

10 $\mu$ M BrdU, and incubated for 48 hours. Cell cultures were then processed as described for Schwann cells.

C6 Rat Glioma Cell Line: Cells, obtained at passage 39, were maintained in DMEM containing 5% FCS, 5% Horse serum (HS), penicillin and streptomycin, at 37°C in a humidified atmosphere of 10% CO<sub>2</sub> in air. Cells were fed or subcultured every three days. For mitogenic assay, cells were plated at a density of 2,000 cells/well in complete medium and incubated for 24 hours. Then medium was replaced with a mixture of 1:1 DMEM and F12 medium containing 0.1% FCS, after washing in serum free medium. Dose responses to GGFs, FCS and  $\alpha$ FGF were then performed and cells were processed through the ELISA as previously described for the other cell types.

PC12 (Rat Adrenal Pheochromocytoma Cells): Cells from ECACC, were maintained in RPMI 1640 supplemented with 10% HS, 5% FCS, penicillin and streptomycin, in collagen coated flasks, at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. Cells were fed every three days by replacing 80% of the medium. For mitogenic assay, cells were plated at a density of 3,000 cells/well in complete medium, on collagen coated plates (50  $\mu$ l/well collagen, Vitrogen Collagen Corp., diluted 1 : 50, 30 min at 37°C) and incubated for 24 hours. The medium was then placed with fresh RPMI either alone or containing 1 mM insulin or 1% FCS. Dose responses to FCS/HS (1:2) as positive control and to GGFs were performed as before. After 48 hours cells were fixed and the ELISA performed as previously described.

III. Results of Mitogenesis Assays: All the experiments presented in this Example were performed using a highly purified sample from a Sepharose 12 chromatography purification step (see Example 1, section D) containing a mixture of GGF-I and GGF-II (GGFs).

First, the results obtained with the BrdU incorporation assay were compared with the classical mitogenic assay for Schwann cells based on [125]I-UdR incorporation into DNA of dividing cells, described by J.P.Brockes (Methods Enzymol. 147:217, 1987).

Figure 13 shows the comparison of data obtained with the two assays, performed in the same cell culture conditions (5,000 cells/well, in 5% FBP/DMEM, incubated in presence of GGFS for 48hrs). As clearly shown, the results are comparable, but BrdU incorporation assay appears to be slightly more sensitive, as suggested by the shift of the curve to the left of the graph, i.e. to lower concentrations of GGFS.

As described under the section "Methods of Mitogenesis Testing", after the immunoreactive BrdU-DNA has been quantitated by reading the intensity of the soluble product of the OPD peroxidase reaction, the original assay plates containing cell monolayers can undergo the second reaction resulting in the insoluble DAB product, which stains the BrdU positive nuclei. The microcultures can then be examined under an inverted microscope, and cell morphology and the numbers of BrdU-positive and negative nuclei can be observed.

In Figure 14a and Figure 14b the BrdU-DNA immunoreactivity, evaluated by reading absorbance at 490 nm, is compared to the number of BrdU-positive nuclei and to the

percentage of BrdU-positive nuclei on the total number of cells per well, counted in the same cultures. Standard deviations were less than 10%. The two evaluation methods show a very good correlation and the discrepancy between the values at the highest dose of GGFs can be explained by the different extent of DNA synthesis in cells detected as BrdU-positive.

The BrdU incorporation assay can therefore provide additional useful information about the biological activity of polypeptides on Schwann cells when compared to the I-UdR incorporation assay. For example, the data reported in Figure 15 show that GGFs can act on Schwann cells to induce DNA synthesis, but at lower doses to increase the number of negative cells present in the microculture after 48 hours.

The assay has then been used on several cell lines of different origin. In Figure 16 the mitogenic responses of Schwann cells and Swiss 3T3 fibroblasts to GGFs are compared; despite the weak response obtained in 3T3 fibroblasts, some clearly BrdU-positive nuclei were detected in these cultures. Control cultures were run in parallel in presence of several doses of FCS or human recombinant PDGF, showing that the cells could respond to appropriate stimuli (not shown).

The ability of fibroblasts to respond to GGFs was further investigated using the BHK 21 C13 cell line. These fibroblasts, derived from kidney, do not exhibit contact inhibition or reach a quiescent state when confluent. Therefore the experimental conditions were designed to have a very low background proliferation without compromising the cell viability. GGFs have a significant mitogenic activity

on BHK21 C13 cells as shown by Figure 17 and Figure 18. Figure 17 shows the BrdU incorporation into DNA by BHK 21 C13 cells stimulated by GGFS in the presence of 0.1% FCS. The good mitogenic response to FCS indicates that cell culture conditions were not limiting. In Figure 18 the mitogenic effect of GGFS is expressed as the number of BrdU-positive and BrdU-negative cells and as the total number of cells counted per well. Data are representative of two experiments run in duplicates; at least three fields per well were counted. As observed for Schwann cells in addition to a proliferative effect at low doses, GGFS also increase the numbers of nonresponding cells surviving. The percentage of BrdU positive cells is proportional to the increasing amounts of GGFS added to the cultures. The total number of cells after 48 hours in presence of higher doses of GGFS is at least doubled, confirming that GGFS induce DNA synthesis and proliferation in BHK21 C13 cells. Under the same conditions, cells maintained for 48 hours in the presence of 2% FCS showed an increase of about six fold (not shown).

C6 glioma cells have provided a useful model to study glial cell properties. The phenotype expressed seems to be dependent on the cell passage, the cells more closely resembling an astrocyte phenotype at an early stage, and an oligodendrocyte phenotype at later stages (beyond passage 70). C6 cells used in these experiments were from passage 39 to passage 52. C6 cells are a highly proliferating population, therefore the experimental conditions were optimized to have a very low background of BrdU incorporation. The presence of 0.1% serum was necessary to maintain cell viability without significantly affecting the

mitogenic responses, as shown by the dose response to FCS (Figure 19).

In Figure 20 the mitogenic responses to aFGF (acidic Fibroblast growth factor) and GGFs are expressed as the percentages of maximal BrdU incorporation obtained in the presence of FCS (8%). Values are averages of two experiments, run in duplicates. The effect of GGFs was comparable to that of a pure preparation of aFGF. aFGF has been described as a specific growth factor for C6 cells (Lim R. et al., Cell Regulation 1:741-746, 1990) and for that reason it was used as a positive control. The direct counting of BrdU positive and negative cells was not possible because of the high cell density in the microcultures. In contrast to the cell lines so far reported, PC12 cells did not show any evident responsiveness to GGFs, when treated under culture conditions in which PC12 could respond to sera (mixture of FCS and HS as used routinely for cell maintenance). Nevertheless the number of cells plated per well seems to affect the behavior of PC12 cells, and therefore further experiments are required.

#### EXAMPLE 4

##### Isolating and Cloning of Nucleotide Sequences encoding proteins containing GGF-I and GGF-II peptides

Isolation and cloning of the GGF-II nucleotide sequences was performed as outlined herein, using peptide sequence information and library screening, and was performed as set out below. It will be appreciated that the peptides of Figures 4 and 5 can be used as the starting point for isolation and cloning of GGF-I sequences by

following the techniques described herein. Indeed, Figure 21, SEQ ID Nos. 54-88) shows possible degenerate oligonucleotide probes for this purpose, and Figure 23, SEQ ID Nos. 90-119, lists possible PCR primers. DNA sequence and polypeptide sequence should be obtainable by this means as with GGF-II, and also DNA constructs and expression vectors incorporating such DNA sequence, host cells genetically altered by incorporating such constructs/vectors, and protein obtainable by cultivating such host cells. The invention envisages such subject matter.

I. Design and Synthesis of oligonucleotide Probes and Primers

Degenerate DNA oligomer probes were designed by backtranslating the amino acid sequences (derived from the peptides generated from purified GGF protein) into nucleotide sequences. Oligomers represented either the coding strand or the non-coding strand of the DNA sequence. When serine, arginine or leucine were included in the oligomer design, then two separate syntheses were prepared to avoid ambiguities. For example, serine was encoded by either TCN or AGY as in 537 and 538 or 609 and 610. Similar codon splitting was done for arginine or leucine (e.g. 544, 545). DNA oligomers were synthesized on a Biosearch 8750 4-column DNA synthesizer using  $\beta$ -cyanoethyl chemistry operated at 0.2 micromole scale synthesis. Oligomers were cleaved off the column (500 angstrom CpG resins) and deprotected in concentrated ammonium hydroxide for 6-24 hours at 55-60°C. Deprotected oligomers were dried under vacuum (Speedvac) and purified by electrophoresis in gels of 15% acrylamide (20 mono : 1 bis), 50 mM Tris-borate-EDTA



buffer containing 7M urea. Full length oligomers were detected in the gels by UV shadowing, then the bands were excised and DNA oligomers eluted into 1.5 mls H<sub>2</sub>O for 4-16 hours with shaking. The eluate was dried, redissolved in  
5 0.1 ml H<sub>2</sub>O and absorbance measurements were taken at 260nm.

Concentrations were determined according to the following formula:

$$(A_{260} \times \text{units/ml}) (60.6/\text{length} = x \mu\text{M})$$

All oligomers were adjusted to 50  $\mu\text{M}$  concentration  
10 by addition of H<sub>2</sub>O.

Degenerate probes designed as above are shown in Figure 21, SEQ ID Nos. 54-88.

PCR primers were prepared by essentially the same procedures that were used for probes with the following  
15 modifications. Linkers of thirteen nucleotides containing restriction sites were included at the 5' ends of the degenerate oligomers for use in cloning into vectors. DNA synthesis was performed at 1 micromole scale using 1,000 angstrom CpG resins and inosine was used at positions where  
20 all four nucleotides were incorporated normally into degenerate probes. Purifications of PCR primers included an ethanol precipitation following the gel electrophoresis purification.

## II. Library Construction and Screening

25 A bovine genomic DNA library was purchased from Stratagene (Catalogue Number: 945701). The library contained  $2 \times 10^6$  15-20kb Sau3A1 partial bovine DNA fragments cloned into the vector lambda DashII. A bovine total brain CDNA library was purchased from Clontech  
30 (Catalogue Number: BL 10139). Complementary DNA libraries

were constructed (In Vitrogen; Stratagene) from mRNA prepared from bovine total brain, from bovine pituitary and from bovine posterior pituitary. In Vitrogen prepared two cDNA libraries: one library was in the vector lambda g10, the other in vector pcDNAI (a plasmid library). The Stratagene libraries were prepared in the vector lambda unizap. Collectively, the cDNA libraries contained 14 million primary recombinant phage.

The bovine genomic library was plated on *E. coli* K12 host strain LE392 on 23 x 23 cm plates (Nunc) at 150,000 to 200,000 phage plaques per plate. Each plate represented approximately one bovine genome equivalent. Following an overnight incubation at 37°C, the plates were chilled and replicate filters were prepared according to procedures of Maniatis et al. (2:60-81). Four plaque lifts were prepared from each plate onto uncharged nylon membranes (Pall Biotyne A or MSI Nitropure). The DNA was immobilized onto the membranes by cross-linking under UV light for 5 minutes or, by baking at 80°C under vacuum for two hours. DNA probes were labelled using T4 polynucleotide kinase (New England Biolabs) with gamma 32P ATP (New England Nuclear; 6500 Ci/mmol) according to the specifications of the suppliers. Briefly, 50 pmols of degenerate DNA oligomer were incubated in the presence of 600 µCi gamma 32P-ATP and 5 units T4 polynucleotide kinase for 30 minutes at 37°C. Reactions were terminated, gel electrophoresis loading buffer was added and then radiolabelled probes were purified by electrophoresis. 32P labelled probes were excised from gel slices and eluted into water. Alternatively, DNA probes were labelled via PCR amplification by incorporation of α-32P-dATP or α-32P dCTP according to the protocol of

Schowalter and Sommer, Anal. Biochem 177:90-94 (1989).  
Probes labelled in PCR reactions were purified by desalting  
on Sephadex G-150 columns.

Prehybridization and hybridization were performed in  
5 GMC buffer (0.52 M NaPi, 7% SDS, 1% BSA, 1.5 mM EDTA, 0.1 M  
NaCl 10 mg/ml tRNA). Washing was performed in oligowash  
(160 ml 1 M Na<sub>2</sub>HPO<sub>4</sub>, 200 ml 20% SDS, 8.0 ml 0.5 M EDTA, 100  
ml 5M NaCl, 3632 ml H<sub>2</sub>O). Typically, 20 filters (400 sq.  
centimeters each) representing replicate copies of ten  
10 bovine genome equivalents were incubated in 200 ml  
hybridization solution with 100 pmols of degenerate  
oligonucleotide probe (128-512 fold degenerate).  
Hybridization was allowed to occur overnight at 5°C below  
the minimum melting temperature calculated for the  
15 degenerate probe. The calculation of minimum melting  
temperature assumes 2°C for an AT pair and 4°C for a GC  
pair.

Filters were washed in repeated changes of oligowash  
at the hybridization temperatures four to five hours and  
20 finally, in 3.2M tetramethylammonium chloride, 1% SDS twice  
for 30 min at a temperature dependent on the DNA probe  
length. For 20mers, the final wash temperature was 60°C.  
Filters were mounted, then exposed to X-ray film (Kodak  
XAR5) using intensifying screens (Dupont Cronex Lightening  
25 Plus). Usually, a three to five day film exposure at minus  
80°C was sufficient to detect duplicate signals in these  
library screens. Following analysis of the results, filters  
could be stripped and reprobbed. Filters were stripped by  
incubating through two successive cycles of fifteen minutes  
30 in a microwave oven at full power in a solution of 1% SDS  
containing 10mM EDTA pH8. Filters were taken through at

least three to four cycles of stripping and reprobing with various probes.

### III. Recombinant Phage Isolation, Growth and DNA Preparation

- 5           These procedures followed standard protocol as described in Recombinant DNA (Maniatis et al 2:60-2:81).

### IV. Analysis of Isolated Clones Using DNA Digestion and Southern Blots

- 10           Recombinant Phage DNA samples (2 micrograms) were digested according to conditions recommended by the restriction endonuclease supplier (New England Biolabs). Following a four hour incubation at 37°C, the reactions products were precipitated in the presence of 0.1M sodium acetate and three volumes of ethanol. Precipitated DNA was
- 15 collected by centrifugation, rinsed in 75% ethanol and dried. All resuspended samples were loaded onto agarose gels (typically 1% in TAE buffer; 0.04M Tris acetate, 0.002M EDTA). Gel runs were at 1 volt per centimeter from 4 to 20 hours. Markers included lambda Hind III DNA fragments
- 20 and/or  $\phi$ X174HaeIII DNA fragments (New England Biolabs). The gels were stained with 0.5 micrograms/ml of ethidium bromide and photographed. For southern blotting, DNA was first depurinated in the gel by treatment with 0.125 N HCl, denatured in 0.5 N NaOH and transferred in 20x SSC (3M
- 25 sodium chloride, 0.03 M sodium citrate) to uncharged nylon membranes. Blotting was done for 6 hours up to 24 hours, then the filters were neutralized in 0.5 Tris HCl pH 7.5, 0.15 M sodium chloride, then rinsed briefly in 50 mM Tris-borate EDTA.

For cross-linking, the filters were wrapped first in transparent plastic wrap, then the DNA side exposed for five minutes to an ultraviolet light. Hybridization and washing was performed as described for library screening (see  
5 section 2 of this Example). For hybridization analysis to determine whether similar genes exist in other species slight modifications were made. The DNA filter was purchased from Clontech (Catalogue Number 7753-1) and contains 5 micrograms of EcoRI digested DNA from various  
10 species per lane. The probe was labelled by PCR amplification reactions as described in section 2 above, and hybridizations were done in 80% buffer B (2 g polyvinylpyrrolidone, 2 g Ficoll-400, 2 g bovine serum albumin, 50 ml 1M Tris-HCl (pH 7.5) 58 g NaCl, 1 g sodium  
15 pyrophosphate, 10 g sodium dodecyl sulfate, 950ml H<sub>2</sub>O) containing 10% dextran sulfate. The probes were denatured by boiling for ten minutes then rapidly cooling in ice water. The probe was added to the hybridization buffer at 10<sup>6</sup> dpm <sup>32</sup>P per ml and incubated overnight at 60°C. The  
20 filters were washed at 60°C first in buffer B followed by 2X SSC, 0.1% SDS then in 1x SSC, 0.1% SDS. For high stringency, experiments, final washes were done in 0.1 x SSC, 1% SDS and the temperature raised to 65°C.

Southern blot data were used to prepare a  
25 restriction map of the genomic clone and to indicate which subfragments hybridized to the GGF probes (candidates for subcloning).

#### V. Subcloning of Segments of DNA Homologous to Hybridization Probes

DNA digests (e.g. 5 micrograms) were loaded onto 1% agarose gels then appropriate fragments excised from the gels following staining. The DNA was purified by adsorption onto glass beads followed by elution using the protocol described by the supplier (Bio 101). Recovered DNA fragments (100-200 ng) were ligated into linearized dephosphorylated vectors, e.g. pT3T7 (Ambion), which is a derivative of pUC18, using T4 ligase (New England Biolabs). This vector carries the *E. coli*  $\beta$  lactamase gene, hence, transformants can be selected on plates containing ampicillin. The vector also supplies  $\beta$ -galactosidase complementation to the host cell, therefore non-recombinants (blue) can be detected using isopropylthiogalactoside and Bluogal (Bethesda Research Labs). A portion of the ligation reactions was used to transform *E. coli* K12 XL1 blue competent cells (Stratagene Catalogue Number: 200236) and then the transformants were selected on LB plates containing 50 micrograms per ml ampicillin. White colonies were selected and plasmid mini preps were prepared for DNA digestion and for DNA sequence analysis. Selected clones were retested to determine if their insert DNA hybridized with the GGF probes.

#### VI. DNA Sequencing

Double stranded plasmid DNA templates were prepared from 5 ml cultures according to standard protocols. Sequencing was by the dideoxy chain termination method using Sequenase 2.0 and a dideoxynucleotide sequencing kit (US Biochemical) according to the manufacturers protocol (a modification of Sanger et al. PNAS; USA 74:5463 (1977)). Alternatively, sequencing was done in a DNA thermal cycler

(Perkin Elmer, model 4800) using a cycle sequencing kit (New England Biolabs; Bethesda Research Laboratories) and was performed according to manufacturers instructions using a 5'-end labelled primer. Sequence primers were either those  
5 supplied with the sequencing kits or were synthesized according to sequence determined from the clones. Sequencing reactions were loaded on and resolved on 0.4mm thick sequencing gels of 6% polyacrylamide. Gels were dried and exposed to X-Ray film. Typically, 35S was incorporated  
10 when standard sequencing kits were used and a 32P end labelled primer was used for cycle sequencing reactions. Sequences were read into a DNA sequence editor from the bottom of the gel to the top (5' direction to 3') and data were analyzed using programs supplied by Genetics Computer  
15 Group (GCG, University of Wisconsin).

#### VII. RNA Preparation and PCR Amplification

Open reading frames detected in the genomic DNA and which contained sequence encoding GGF peptides were extended via PCR amplification of pituitary RNA. RNA was prepared  
20 from frozen bovine tissue (Pelfreeze) according to the guanidine neutral-CsCl procedure (Chirgwin et. al. Biochemistry 18:5294 (1979).) Polyadenylated RNA was selected by oligo-dT cellulose column chromatography (Aviv and Leder PNAS (USA) 69:1408 (1972)).

25 Specific DNA target sequences were amplified beginning with either total RNA or polyadenylated RNA samples that had been converted to cDNA using the Perkin Elmer PCR/RNA Kit Number: N808-0017. First strand reverse transcription reactions used 1 µg template RNA and either  
30 primers of oligo dT with restriction enzyme recognition site

linkers attached or specific antisense primers determined from cloned sequences with restriction sites attached. To produce the second strand, the primers either were plus strand unique sequences as used in 3' RACE reactions (Frohman et. al., PNAS (USA) 85:8998 (1988)) or were oligo dT primers with restriction sites attached if the second target site had been added by terminal transferase tailing first strand reaction products with dATP (e.g. 5' race reactions, Frohman et. al., *ibid*). Alternatively, as in anchored PCR reactions the second strand primers were degenerate, hence, representing particular peptide sequences.

The amplification profiles followed the following general scheme: 1) five minutes soak file at 95°C; 2) thermal cycle file of 1 minute, 95°C; 1 minute ramped down to an annealing temperature of 45°C, 50°C or 55°C; maintain the annealing temperature for one minute; ramp up to 72°C over one minute; extend at 72°C for one minute or for one minute plus a 10 second auto extension; 3) extension cycle at 72°C, five minutes, and; 4) soak file 4°C for infinite time. Thermal cycle files (#2) usually were run for 30 cycles. A sixteen µl sample of each 100 µl amplification reaction was analyzed by electrophoresis in 2% Nusieve 1% agarose gels run in TAE buffer at 4 volts per centimeter for three hours. The gels were stained, then blotted to uncharged nylon membranes which were probed with labelled DNA probes that were internal to the primers.

Specific sets of DNA amplification products could be identified in the blotting experiments and their positions used as a guide to purification and reamplification. When appropriate, the remaining portions of selected samples were



loaded onto preparative gels, then following electrophoresis four to five slices of 0.5 mm thickness (bracketing the expected position of the specific product) were taken from the gel. The agarose was crushed, then soaked in 0.5 ml of electrophoresis buffer from 2-16 hours at 40°C. The crushed agarose was centrifuged for two minutes and the aqueous phase was transferred to fresh tubes.

Reamplification was done on five microliters (roughly 1% of the product) of the eluted material using the same sets of primers and the reaction profiles as in the original reactions. When the reamplification reactions were completed, samples were extracted with chloroform and transferred to fresh tubes. Concentrated restriction enzyme buffers and enzymes were added to the reactions in order to cleave at the restriction sites present in the linkers. The digested PCR products were purified by gel electrophoresis, then subcloned into vectors as described in the subcloning section above. DNA sequencing was done described as above.

#### VIII. DNA Sequence Analysis

DNA sequences were assembled using a fragment assembly program and the amino acid sequences deduced by the GCG programs GelAssemble, Map and Translate. The deduced protein sequences were used as a query sequence to search protein sequence databases using WordSearch. Analysis was done on a VAX Station 3100 workstation operating under VMS 5.1. The database search was done on SwissProt release number 21 using GCG Version 7.0.

IX. Results of Cloning and Sequencing of genes encoding GGF-I and GGF-II

As indicated above, to identify the DNA sequence encoding bovine GGF-II degenerate oligonucleotide probes were designed from GGF-II peptide sequences. GGF-II 12 (SEQ ID No. 44), a peptide generated via lysyl endopeptidase digestion of a purified GGF-II preparation (see Figures 11 and 12) showed strong amino acid sequence homology with GGF-I 07 (SEQ ID No. 39), a tryptic peptide generated from a purified GGF-I preparation. GGF-II 12 was thus used to create ten degenerate oligonucleotide probes (see oligos 609, 610 and 649 to 656 in Figure 21, SEQ ID Nos. 69, 70, 71 and 79, respectively). A duplicate set of filters were probed with two sets (set 1=609, 610; set 2=649-656) of probes encoding two overlapping portions of GGF-II 12. Hybridization signals were observed, but, only one clone hybridized to both probe sets. The clone (designated GGF2BG1) was purified.

Southern blot analysis of DNA from the phage clone GGF2BG1 confirmed that both sets of probes hybridized with that bovine DNA sequence, and showed further that both probes reacted with the same set of DNA fragments within the clone. Based on those experiments a 4 kb Eco RI sub-fragment of the original clone was identified, subcloned and partially sequenced. Figure 22 shows the nucleotide sequence, SEQ ID No. 89) and the deduced amino acid sequence of the initial DNA sequence readings that included the hybridization sites of probes 609 and 650, and confirmed that a portion of this bovine genomic DNA encoded peptide 12 (KASLADSGEYM).

Further sequence analysis demonstrated that GGF-II  
12 resided on a 66 amino acid open reading frame (see below)  
which has become the starting point for the isolation of  
overlapping sequences representing a putative bovine GGF-II  
5 gene and a cDNA.

Several PCR procedures were used to obtain  
additional coding sequences for the putative bovine GGF-II  
gene. Total RNA and oligo dT-selected (poly A containing)  
RNA samples were prepared from bovine total pituitary,  
10 anterior pituitary, posterior pituitary, and hypothalamus.  
Using primers from the list shown in Figure 23, SEQ ID Nos.  
109-119, one-sided PCR reactions (RACE) were used to amplify  
cDNA ends in both the 3' and 5' directions, and anchored PCR  
reactions were performed with degenerate oligonucleotide  
15 primers representing additional GGF-II peptides. Figure 24  
summarizes the contiguous DNA structures and sequences  
obtained in those experiments. From the 3' RACE reactions,  
three alternatively spliced cDNA sequences were produced,  
which have been cloned and sequenced. A 5' RACE reaction  
20 led to the discovery of an additional exon containing coding  
sequence for at least 52 amino acids. Analysis of that  
deduced amino acid sequence revealed peptides GGF-II-6 and a  
sequence similar to GGF-I-18 (see below). The anchored PCR  
reactions led to the identification of (cDNA) coding  
25 sequences of peptides GGF-II-1, 2, 3 and 10 contained within  
an additional cDNA segment of 300 bp. The 5' limit of this  
segment (i.e., segment E, see Fig. 31) is defined by the  
oligonucleotide which encodes peptide GGF-II-1 and which was  
used in the PCR reaction (additional 5' sequence data exists  
30 as described for the human clone in Example 6). Thus this

clone contains nucleotide sequences encoding six out of the existing total of nine novel GGF-II peptide sequences.

The cloned gene was characterized first by constructing a physical map of GGF2BG1 that allowed us to position the coding sequences as they were found (see below, Figure 25). DNA probes from the coding sequences described above have been used to identify further DNA fragments containing the exons on this phage clone and to identify clones that overlap in both directions. The putative bovine GGF-II gene is divided into at least 5 coding segments. Coding segments are defined as discrete lengths of DNA sequence which can be translated into polypeptide sequences using the universal genetic code. The coding segments described in Figure 31 and referred to in the present application are: 1) particular exons present within the GGF gene (e.g. coding segment a), or 2) derived from sets of two or more exons that appear in specific subgroups of mRNAs, where each set can be translated into the specific polypeptide segments as in the gene products shown. The polypeptide segments referred to in the claims are the translation products of the analogous DNA coding segments. Only coding segments A and B have been defined as exons and sequenced and mapped thus far. The summary of the contiguous coding sequences identified is shown in Figure 26. The exons are listed (alphabetically) in the order of their discovery. It is apparent from the intron/exon boundaries that exon B may be included in cDNAs that connect coding segment E and coding segment A. That is, exon B cannot be spliced out without compromising the reading frame. Therefore, we suggest that three alternative splicing patterns can produce putative bovine

GGF-II cDNA sequences 1, 2 and 3. The coding sequences of these, designated GGF2BPP1.CDS, GGF2BPP2.CDS and GGF2BPP3.CDS, respectively, are given in Figures 28a (SEQ ID No. 133), 28b (SEQ ID No. 134), and 28c (SEQ ID No. 135), respectively. The deduced amino acid sequence of the three cDNAs is also given in Figures 28a, (SEQ ID No. 133), 28b (SEQ ID No. 134), and 28c (SEQ ID No. 135).

The three deduced structures encode proteins of lengths 206, 281 and 257 amino acids. The first 183 residues of the deduced protein sequence are identical in all three gene products. At position 184 the clones differ significantly. A codon for glycine GGT in GGF2BPP1 also serves as a splice donor for GGF2BPP2 and GGF2BPP3, which alternatively add on exons C, C/D, C/D' and D or C, C/D and D, respectively, and shown in figure 33, SEQ ID No. 149). GGF1IBPP1 is a truncated gene product which is generated by reading past the coding segment A splice junction into the following intervening sequence (intron). This represents coding segment A' in figure 31 (SEQ ID No. 140). The transcript ends adjacent to a canonical AATAAA polyadenylation sequence, and we suggest that this truncated gene product represents a bona fide mature transcript. The other two longer gene products share the same 3' untranslated sequence and polyadenylation site.

All three of these molecules contain six of the nine novel GGF-II peptide sequences (see Figure 12) and another peptide is highly homologous to GGF-I-18 (see Figure 27). This finding gives a high probability that this recombinant molecule encodes at least a portion of bovine GGF-II. Furthermore, the calculated isoelectric points for the three peptides are consistent with the physical properties of

GGF-I and II. Since the molecular size of GGF-II is roughly 60 kD, the longest of the three cDNAs should encode a protein with nearly one-half of the predicted number of amino acids.

5 A probe encompassing the B and A exons was labelled via PCR amplification and used to screen a cDNA library made from RNA isolated from bovine posterior pituitary. One clone (GGF2BPP5) showed the pattern indicated in figure 30 and contained an additional DNA coding segment (G) between  
10 coding segments A and C. The entire nucleic acid sequence is shown in figure 32 (SEQ ID No. 148). The predicted translation product from the longest open reading frame is 241 amino acids. A portion of a second cDNA (GGF2BPP4) was also isolated from the bovine posterior pituitary library  
15 using the probe described above. This clone showed the pattern indicated in figure 30. This clone is incomplete at the 5' end, but is a splicing variant in the sense that it lacks coding segments G and D. BPP4 also displays a novel 3' end with regions H, K and L beyond region C/D. The  
20 sequence of BPP4 is shown in figure 34 (SEQ ID No. 150).

#### EXAMPLE 5

##### GGF Sequences in Various Species

Database searching has not revealed any meaningful similarities between any predicted GGF translation products  
25 and known protein sequences. This suggests that GGF-II is the first member of a new family or superfamily of proteins. In high stringency cross hybridization studies (DNA blotting experiments) with other mammalian DNAs we have shown, clearly, that DNA probes from this bovine recombinant  
30 molecule can readily detect specific sequences in a variety

of samples tested. A highly homologous sequence is also detected in human genomic DNA. The autoradiogram is shown in figure 29. The signals in the lanes containing rat and human DNA represent the rat and human equivalents of the GGF gene, the sequences of several cDNA's encoded by this gene have been recently reported by Holmes et al. (Science 256: 1205 (1992)) and Wen et al. (Cell 69: 559 (1992)).

#### EXAMPLE 6

##### Isolation of a Human Sequence Encoding Human GGF2

10 Several human clones containing sequences from the bovine GGFII coding segment E were isolated by screening a human cDNA library prepared from brain stem (Stratagene catalog #935206). This strategy was pursued based on the strong link between most of the GGF2 peptides (unique to  
15 GGF2) and the predicted peptide sequence from clones containing the bovine E segment. This library was screened as described in Example 4, Section II using the oligonucleotide probes 914-919 listed below.

914TCGGGCTCCATGAAGAAGATGTA

20 915TCCATGAAGAAGATGTACCTGCT

916ATGTACCTGCTGTCCTCCTTGA

917TTGAAGAAGGACTCGCTGCTCA

918AAAGCCGGGGGCTTGAAGAA

919ATGARGTGTGGGCGGCGAAA

25 Clones detected with these probes were further analyzed by hybridization. A probe derived from coding segment A (see Figure 21), which was produced by labeling a polymerase chain reaction (PCR) product from segment A, was also used to screen the primary library. Several clones  
30 that hybridized with both A and E derived probes were

selected and one particular clone, GGF2HBS5, was selected for further analysis. This clone is represented by the pattern of coding segments (EBACC/D'D as shown in Figure 31). The E segment in this clone is the human equivalent of the truncated bovine version of E shown in Figure 37.

5 GGF2HBS5 is the most likely candidate to encode GGF-II of all the "putative" GGF-II candidates described. The length of coding sequence segment E is 786 nucleotides plus 264 bases of untranslated sequence. The predicted size of the protein encoded by GGF2HBS5 is approximately 423 amino acids (approximately 45 kilodaltons, see Figure 45, SEQ ID NO: 167), which is similar to the size of the deglycosylated form of GGF-II (see Example 16). Additionally, seven of the GGF-II peptides listed in Figure 27 have equivalent

15 sequences which fall within the protein sequence predicted from region E. Peptides II-6 and II-12 are exceptions, which fall in coding segment B and coding segment A, respectively. RNA encoding the GGF2HBS5 protein was produced in an in vitro transcription system driven by the bacteriophage T7 promoter resident in the vector (Bluescript SK [Stratagene Inc.] see Figure 44) containing the GGF2HBS5 insert. This RNA was translated in a cell free (rabbit reticulocyte) translation system and the size of the protein product was 45 Kd. Additionally, the cell-free product has

20 been assayed in a Schwann cell mitogenic assay to confirm biological activity. Schwann cells treated with conditioned medium show both increased proliferation as measured by incorporation of <sup>125</sup>I-Uridine and phosphorylation on tyrosine of a protein in the 185 kilodalton range.

25

30 Thus the size of the product encoded by GGF2HBS5 and the presence of DNA sequences which encode human peptides



highly homologous to the bovine peptides shown in Figure 12 confirm that GGF2HBS5 encodes the human equivalent of bovine GGF2. The fact that conditioned media prepared from cells transformed with this clone elicits Schwann cell mitogenic activity confirms that the GGFIIHBS5 gene produce (unlike the BPP5 gene product) is secreted. Additionally the GGFIIHBS5 gene product seems to mediate the Schwann cell proliferation response via a receptor tyrosine kinase such as p185<sup>erbB2</sup> or a closely related receptor (see Example 14).

10 EXAMPLE 7

Expression of Human Recombinant GGF2 in Mammalian and Insect Cells

The GGF2HBS5 cDNA clone encoding human GGF2 (as described in Example 6 and also referred to herein as HBS5) was cloned into vector pcDL-SR $\alpha$ 296 (Takebe et al. Mol. Cell. Biol. 8:466-472 (1988) and COS-7 cells were transfected in 100 mm dishes by the DEAE-dextran method (Sambrook et al. Molecular Cloning: A Laboratory Manual 2nd ed. CSH Laboratory NY (1989). Cell lysates or conditioned media from transiently expressing COS cells were harvested at 3 or 4 days post-transfection. To prepare lysates, cell monolayers were washed with PBS, scraped from the dishes lysed by three freeze/thaw cycles in 150  $\mu$ l of 0.25 M Tris-HCl, pH8. Cell debris was pelleted and the supernatant recovered. Conditioned media samples (7 ml.) were collected, then concentrated and buffer exchanged with 10 mM Tris, pH 7.4 using Centiprep-10 and Centricon-10 units as described by the manufacturer (Amicon, Beverly, MA). Rat nerve Schwann cells were assayed for incorporation of DNA synthesis precursors, as described (see Example 3).

Conditioned media or cell lysate samples were tested in the Schwann cell proliferation assay as described in Example 3. The mitogenic activity data are shown in Fig. 46. The cDNA, GGF2HBS5, encoding GGF2 directed the secretion of the protein product to the medium. A small proportion of total activity was detectable inside the cells as determined by assays using cell lysates. GGF2HFB1 and GGFBPP5 cDNA's failed to direct the secretion of the product to the extracellular medium. GGF activity from these clones was detectable only in cell lysates (Fig. 46).

Recombinant GGF2 was also expressed in CHO cells. The GGF2HBS5 cDNA encoding GGF2 was cloned into the EcoRI site of vector pcdhfrpolyA (Fig. 54) and transfected into the DHFR negative CHO cell line (DG44) by the calcium phosphate coprecipitation method (Graham and Van Der Eb, Virology 52:456-467 (1973). Clones were selected in nucleotide and nucleoside free  $\alpha$  medium (Gibco) in 96-well plates. After 3 weeks, conditioned media samples from individual clones were screened for expression of GGF by the Schwann cell proliferation assay as described in Example 3. Stable clones which secreted significant levels of GGF activity into the medium were identified. Schwann cell proliferation activity data from different volume aliquots of CHO cell conditioned medium were used to produce the dose response curve shown in Fig. 47 (Graham and Van Der Eb, Virology 52:456, 1973). This material was analyzed on a Western blot probed with polyclonal antisera raised against a GGF2 specific peptide. A broad band of approximately 69-90 Kd (the expected size of GGF2 extracted from pituitary and higher molecular weight glycoforms) is specifically labeled (Fig. 49, lane 12).

Recombinant GGF2 was also expressed in insect cells using Baculovirus expression. Sf9 insect cells were infected with baculovirus containing the GGF2HBS5 cDNA clone at a multiplicity of 3-5 ( $10^6$  cells/ml) and cultured in  
5 Sf900-II medium (Gibco). Schwann cell mitogenic activity was secreted into the extracellular medium (Fig. 48). Different volumes of insect cell conditioned medium were tested in the Schwann cell proliferation assay in the absence of forskolin and the data used to produce the dose  
10 response curve shown in Fig. 48.

This material was also analyzed on a Western blot (Fig. 47) probed with the GGF II specific antibody described above. A band of 45 Kd, the size of deglycosylated GGF-II (see Example 16) was seen.

15 The methods used in this example were as follows:  
Schwann cell mitogenic activity of recombinant human and bovine glial growth factors was determined as follows: Mitogenic responses of cultured Schwann cells were measured in the presence of 5  $\mu$ M forskolin using crude recombinant  
20 GGF preparations obtained from transient mammalian expression experiments. Incorporation of [ $^{125}$ I]-Uridine was determined following an 18-24 hour exposure to materials obtained from transfected or mock transfected COS cells as described in the Methods. The mean and standard deviation  
25 of four sets of data are shown. The mitogenic response to partially purified native bovine pituitary GGF (carboxymethyl cellulose fraction; Goodearl et al., submitted) is shown (GGF) as a standard of one hundred percent activity.

30 cDNAs (Fig. 53) were cloned into pcDL-SR $\alpha$ 296 (Takebe et al., Mol. Cell Biol. 8:466-472 (1988)), and COS-7 cells

were transfected in 100 mm dishes by the DEAE-dextran method (Sambrook et al., In *Molecular Cloning. A Laboratory Manual*, 2nd. ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989)). Cell lysates or conditioned media were harvested at 3 or 4 days post-transfection. To prepare lysates, cell monolayers were washed with PBS, scraped from the dishes, and lysed by three freeze/thaw cycles in 150  $\mu$ l of 0.25 M Tris-HCl, pH 8. Cell debris was pelleted and the supernatant recovered. Conditioned media samples (7 mls) were collected, then concentrated and buffer exchanged with 10 mM Tris, pH 7.4 using Centriprep-10 and Centricon-10 units as described by the manufacturer (Amicon, Beverly, MA). Rat sciatic nerve Schwann cells were assayed for incorporation of DNA synthesis precursors, as described (Davis and Stroobant, J. Cell Biol. 110:1353-1360 (1990); Brookes et al., Brain Res. 165:105-118 (1979)).

Western blots of recombinant CHO cell conditioned medium were performed as follows: A recombinant CHO clone was cultured in 7 ml. of MCDB302 protein-free medium for 3 days. 2 ml of conditioned medium was concentrated, buffered exchanged against 10 mM Tris-HCl, pH 7.4 and lyophilized to dryness. The pellet was resuspended in SDS-PAGE sample buffer, subjected to reducing SDS gel electrophoresis and analyzed by Western blotting with a GGF peptide antibody. A CHO control was done by using conditioned medium from untransfected CHO-DG44 host and the CHO HBS5 levels were assayed using conditioned medium from a recombinant clone.

#### EXAMPLE 8

#### Isolation of Other Human Sequences Related to Bovine GGF

The result in Examples 5 and 6 indicate that GGF related sequences from human sources can also be easily isolated by using DNA probes derived from bovine GGF sequences. Alternatively the procedure described by Holmes et al. (Science 256: 1205 (1992)) can be used. In this example a human protein (heregulin  $\alpha$ ), which binds to and activates the p185<sup>erbB2</sup> receptor (and is related to GGF), is purified from a tumor cell line and the derived peptide sequence is used to produce oligonucleotide probes which were utilized to clone the cDNA's encoding heregulin. The biochemical assay for p185<sup>erbB2</sup> receptor activation is distinguished from Schwann cell proliferation. This is a similar approach to that used in examples 1-4 for the cloning of GGF sequences from pituitary cDNAs. The heregulin protein and complementary DNAs were isolated from tumor cell lines according to the following procedures. Heregulin was purified from medium conditioned by MDA-MB-231 breast cancer cells (ATCC #HTB 26) grown on Percell Biolytica microcarrier beads (Hyclone Labs). The medium (10 liters) was concentrated ~25-fold by filtration through a membrane (10-kD cutoff) (Millipore) and clarified by centrifugation and filtration through a filter (0.22  $\mu$ m). The filtrate was applied to a heparin Sepharose column (Pharmacia) and the proteins were eluted with steps of 0.3, 0.6, and 0.9 M NaCl in phosphate-buffered saline. Activity in the various chromatographic fractions was measured by quantifying the increase in tyrosine phosphorylation of p185<sup>erbB2</sup> in MCF-7 breast tumor cells (ATCC # HTB 22). MCF-7 cells were plated in 24-well Costar plates in F12 (50%) Dulbecco's minimum essential medium (50%) containing serum (10%) (10<sup>5</sup> cells per well), and allowed to attach for at

least 24 hours. Prior to assay, cells were transferred into medium without serum for a minimum of 1 hour. Column fractions (10 to 100  $\mu$ l) were incubated for 30 min. at 37°. Supernatants were then aspirated and the reaction was

5 stopped by the addition of SDS-PAGE sample buffer 100  $\mu$ l). Samples were heated for 5 min. at 100°C, and portions (10 to 15  $\mu$ l) were applied to a tris-glycine gel (4 to 20%) (Novex). After electrophoresis, proteins were electroblotted onto a polyvinylidenedifluoride (PVDF)

10 membrane and then blocked with bovine serum albumin (5%) in tris-buffered saline containing Tween-20 (0.05%) (TBST). Blots were probed with a monoclonal antibody (1:1000 dilution) to phosphotyrosine (Upstate Biotechnology) for a minimum of 1 hour at room temperature. Blots were washed

15 with TBST, probed with an antibody to mouse immunoglobulin G conjugated to alkaline phosphatase (Promega) (diluted 1:7500) for a minimum of 30 min. at room temperature. Reactive bands were visualized with

20 5-bromo-4-chloro-3-indoyl-1-phosphate and nitro-blue tetrazolium. Immunoblots were scanned with a Scan Jet Plus (Hewlett-Packard) densitometer. Signal intensities for unstimulated MCF-7 cells were 20 to 30 units. Fully stimulated p185<sup>erbB2</sup> yielded signals of 180 to 200 units. The 0.6 M NaCl pool, which contained most of the activity, was

25 applied to a polyaspartic acid (PolyLC) column equilibrated in 17 mM sodium phosphate (pH 6.8) containing ethanol (30%). A linear gradient from 0.3 M to 0.6 M NaCl in the equilibration buffer was used to elute bound proteins. A peak of activity (at ~0.45 M NaCl) was further fractionated

30 on a C4 reversed-phase column (SynChropak RP-4) equilibrated in buffer containing TFA (0.1%) and acetonitrile (15%).

Proteins were eluted from this column with an acetonitrile gradient from 25 to 40% over 60 min. Fractions (1 ml) were collected, assayed for activity, and analyzed by SDS-PAGE on tris-glycine gels (4-20%, Novex).

- 5 HPLC-purified HRG- $\alpha$  was digested with lysine C in SDS (0.1%), 10 mM dithiothreitol, 0.1 M  $\text{NH}_4\text{HCO}_3$  (pH 8.0) for 20 hours at 37°C and the resultant fragments were resolved on a Synchrom C4 column (4000Å<sup>2</sup>, 0.2 by 10 cm). The column was equilibrated in 0.1% TFA and eluted with a 1-propanol
- 10 gradient in 0.1% TFA (W. J. Henzel, J. T. Stults, C. Hsu, D. W. Aswad, *J. Biol. Chem.* 264, 15905 (1989)). Peaks from the chromatographic run were dried under vacuum and sequenced. One of the peptides (eluting at ~24% 1-propanol) gave the sequence [A]AEKEKTF[C]VNGGEXFMVKDLXNP (SEQ ID No. 162).
- 15 Residues in brackets were uncertain and an X represents a cycle in which it was not possible to identify the amino acid. The initial yield was 8.5 pmol and the sequence did not correspond to any known protein. Residues 1, 9, 15, and 22 were later identified in the cDNA sequence as cysteine.
- 20 Direct sequencing of the ~45-kD band from a gel that had been overloaded and blotted onto a PVDF membrane revealed a low abundance sequence XEXKE[G][R]GK[G]K[G]KKKEXGXG[K] (SEQ ID No. 30) with a very low initial yield (0.2 pmol). This corresponded to amino acid residues 2 to 22 of heregulin- $\alpha$
- 25 (Fig. 31), suggesting that serine 2 is the  $\text{NH}_2$ -terminus of proHRG- $\alpha$ . Although the  $\text{NH}_2$  terminus was blocked, it was observed that occasionally a small amount of a normally blocked protein may not be post-translationally modified. The  $\text{NH}_2$  terminal assignment was confirmed by mass
- 30 spectrometry of the protein after digestion with cyanogen bromide. The COOH-terminus of the isolated protein has not

been definitely identified; however, by mixture sequencing of proteolytic digests, the mature sequence does not appear to extend past residue 241. Abbreviations for amino residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. As a source of cDNA clones, an oligo(dT)-primed  $\lambda$ gt10 (T. V. Huynn, R. A. Young, R. W. Davis,  $\lambda$ gt10 and  $\lambda$ gt11 DNA Cloning Techniques: A Practical Approach, D. Glover, Ed. (IRC Press, Oxford, (1984)) cDNA library was constructed (U. Gubler and B. J. Hoffman, Gene 25, 263 (1983)) with mRNA purified (J. M. Chirwin, A. E. Przbyla, R. J. MacDonald, W. J. Rutter, Biochemistry 18, 5294 (1979)) from MDA-MB-231 cells. The following eightfold degenerate antisense deoxyoligonucleotide encoding the 13-amino acid sequence AEKEKTFVCVNGGE (SEQ ID No. 31)(13) was designed on the basis of human codon frequency optima (R. Lathe, J. Mol. Biol. 183, 1 (1985)) and chemically synthesized:  
5'-CTCGCC (G OR T) CC (A OR G) TTCAC (A OR G) CAGAAGGTCTTCTCCTTCTCAGC-3' (SEQ ID No. 40). For the purpose of probe design a cysteine was assigned to an unknown residue in the amino acid sequence. The probe was labeled by phosphorylation and hybridized under low-stringency conditions to the cDNA library. The proHRG- $\alpha$  protein was identified in this library. HRB- $\beta$ 1 cDNA was identified by probing a second oligo(dT)-primed  $\lambda$ gt10 library made from MDA-MB-231 cell mRNA with sequences derived from both the 5' and 3' ends of proHRG- $\alpha$ . Clone 13 (Fig. 2A) was a product of screening a primed (5'-CCTCGCTCCTTCTTCTTGGCCCTTC-3' primer (SEQ ID No. 41); proHRG- $\alpha$  antisense nucleotides 33 to 56) MDA-MB-231  $\lambda$ gt10 library with 5' HRG- $\alpha$  sequence. A sequence



corresponding to the 5' end of clone 13 as the probe was used to identify proHRG $\beta$ 2 and proHRG $\beta$ 3 in a third oligo(dT)-primed  $\lambda$ gt10 library derived from MDA-MB-231 cell mRNA. Two cDNA clones encoding each of the four HRGs were  
5 sequenced (F. Sanger, S. Milken, A. R. Coulson, Proc. Natl. Acad. Sci. U.S.A. 74, 5463 1977]). Another cDNA designated clone 84 has an amino acid sequence identical to proHRG $\beta$ 2 through amino acid 420. A stop codon at position 421 is followed by a different 3'-untranslated sequence.

10

EXAMPLE 9Isolation of a Further Splicing Variant

The methods in Example 6 produced four closely related sequences (heregulin  $\alpha$ ,  $\beta$ 1,  $\beta$ 2,  $\beta$ 3) which arise as a result of splicing variation. Peles et al. (Cell 69, 205  
15 (1992)), and Wen et al. (Cell 69, 559 (1992)) have isolated another splicing variant (from rat) using a similar purification and cloning approach to that described in Examples 1-4 and 6 involving a protein which binds to p185<sup>erbB2</sup>. The cDNA clone was obtained as follows (via the  
20 purification and sequencing of a p185<sup>erbB2</sup> binding protein from a transformed rat fibroblast cell line).

A p185<sup>erbB2</sup> binding protein was purified from conditioned medium as follows. Pooled conditioned medium from three harvests of 500 roller bottles (120 liters total)  
25 was cleared by filtration through 0.2  $\mu$  filters and concentrated 31-fold with a Pelicon ultrafiltration system using membranes with a 20kd molecular size cutoff. All the purification steps were performed by using a Pharmacia fast protein liquid chromatography system. The concentrated

material was directly loaded on a column of heparin-Sepharose (150 ml, preequilibrated with phosphate-buffered saline (PBS)). The column was washed with PBS containing 0.2 M NaCl until no absorbance at 280 nm wavelength could be detected. Bound proteins were then eluted with a continuous gradient (250 ml) of NaCl (from 0.2 M to 1.0 M), and 5 ml fractions were collected. Samples (0.01 ml of the collected fractions were used for the quantitative assay of the kinase stimulatory activity.

Active fractions from three column runs (total volume = 360 ml) were pooled, concentrated to 25 ml by using a YM10 ultrafiltration membrane (Amicon, Danvers, MA), and ammonium sulfate was added to reach a concentration of 1.7 M. After clearance by centrifugation (10,000 x g, 15 min.), the pooled material was loaded on a phenyl-Superose column (HR10/10, Pharmacia). The column was developed with a 45 ml gradient of  $(\text{NH}_4)_2\text{SO}_4$  (from 1.7 M to no salt) in 0.1 M  $\text{Na}_2\text{PO}_4$  (pH 7.4), and 2 ml fractions were collected and assayed (0.002 ml per sample) for kinase stimulation (as described in Example 6). The major peak of activity was pooled and dialyzed against 50 mM sodium phosphate buffer (pH 7.3). A Mono-S cation-exchange column (HR5/5, Pharmacia) was preequilibrated with 50 mM sodium phosphate. After loading the active material (0.884 mg of protein; 35 ml), the column was washed with the starting buffer and then developed at a rate of 1 ml/min. with a gradient of NaCl. The kinase stimulatory activity was recovered at 0.45-0.55 M salt and was spread over four fractions of 2 ml each. These were pooled and loaded directly on a  $\text{Cu}^{+2}$  chelating columns (1.6 ml, HR2/5 chelating Superose, Pharmacia). Most of the proteins adsorbed to the resin, but they gradually eluted

with a 30 ml linear gradient of ammonium chloride (0-1 M). The activity eluted in a single peak of protein at the range of 0.05 to 0.2 M  $\text{NH}_4\text{Cl}$ . Samples from various steps of purification were analyzed by gel electrophoresis followed by silver staining using a kit from ICN (Costa Mesa, CA), and their protein contents were determined with a Coomassie blue dye binding assay using a kit from Bio-Rad (Richmond, CA).

The p44 protein (10  $\mu\text{g}$ ) was reconstituted in 200  $\mu\text{l}$  of 0.1 M ammonium bicarbonate buffer (pH 7.8). Digestion was conducted with L-1-tosyl-amide 2-phenylethyl chloromethyl ketone-treated trypsin (Serva) at 37°C for 18 hr. at an enzyme-to-substrate ratio of 1:10. The resulting peptide mixture was separated by reverse-phase HPLC and monitored at 215 nm using a Vydac C4 micro column (2.1 mm i.d. x 15 cm, 300 Å) and an HP 1090 liquid chromatographic system equipped with a diode-array detector and a workstation. The column was equilibrated with 0.1% trifluoroacetic acid (mobile phase A), and elution was effected with a linear gradient from 0%-55% mobile phase B (90% acetonitrile in 0.1% trifluoroacetic acid) over 70 min. The flow rate was 0.2 ml/min. and the column temperature was controlled at 25°C. One-third aliquots of the peptide peaks collected manually from the HPLC system were characterized by N-terminal sequence analysis by Edman degradation. The fraction eluted after 27.7 min. (T27.7) contained mixed amino acid sequences and was further rechromatographed after reduction as follows: A 70% aliquot of the peptide fraction was dried in vacuo and reconstituted in 100  $\mu\text{l}$  of 0.2 M ammonium bicarbonate buffer (pH 7.8). DTT (final concentration 2 mM) was added to the solution, which was

then incubated at 37°C for 30 min. The reduced peptide mixture was then separated by reverse-phase HPLC using a Vydac column (2.1 mm i.d. x 15 cm). Elution conditions and flow rate were identical to those described above. Amino acid sequence analysis of the peptide was performed with a Model 477 protein sequencer (Applied Biosystems, Inc., Foster City, CA) equipped with an on-line phenylthiohydantoin (PTH) amino acid analyzer and a Model 900 data analysis system (Hunkapiller et al. (1986) In Methods of Protein Microcharacterization, J.E. Shively, ed. (Clifton, New Jersey: Humana Press p. 223-247). The protein was loaded onto a trifluoroacetic acid-treated glass fiber disc precycled with polybrene and NaCl. The PTH-amino acid analysis was performed with a micro liquid chromatography system (Model 120) using dual syringe pumps and reverse-phase (C-18) narrow bore columns (Applied Biosystems, 2.1 mm x 250 mm).

RNA was isolated from Rat1-EJ cells by standard procedures (Maniatis et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York (1982) and poly (A)<sup>+</sup> was selected using an mRNA Separator kit (Clontech Lab, Inc., Palo Alto, CA). cDNA was synthesized with the Superscript kit (from BRL Life Technologies, Inc., Bethesda, MD). Column-fractionated double-strand cDNA was ligated into an SalI- and NotI-digested pJT-2 plasmid vector, a derivative of the pCD-X vector (Okayama and Berg, Mol. Cell Biol. 3: 280 (1983)) and transformed into DH10B *E. coli* cells by electroporation (Dower et al., Nucl. Acids Res. 16: 6127 (1988)). Approximately 5 x 10<sup>5</sup> primary transformants were screened with two oligonucleotide probes that were derived from the protein sequences of the N-terminus of NDF

(residues 5-24) and the T40.4 tryptic peptide (residues 7-12). Their respective sequences were as follows (N indicates all 4 nt):

(1) 5'-ATA GGG AAG GGC GGG GGA AGG GTC NCC CTC NGC  
           A      T  
           AGG GCC GGG CTT GCC TCT GGA GCC TCT-3'  
 (2) 5'-TTT ACA CAT ATA TTC NCC-3'  
           C      G          G      C

(1: SEQ ID No. 167; 2: SEQ ID No. 168)

10 The synthetic oligonucleotides were end-labeled with  
 [γ-<sup>32</sup>P]ATP with T4 polynucleotide kinase and used to screen  
 replicate sets of nitrocellulose filters. The hybridization  
 solution contained 6 x SSC, 50 mM sodium phosphate (pH 6.8),  
 0.1% sodium pyrophosphate, 2 x Denhardt's solution, 50 µg/ml  
 15 salmon sperm DNA, and 20% formamide (for probe 1) or no  
 formamide (for probe 2). The filters were washed at either  
 50°C with 0.5 x SSC, 0.2% SDS, 2 mM EDTA (for probe 1) or at  
 37°C with 2 x SSC, 0.2% SDS, 2 mM EDTA (for probe 2).  
 Autoradiography of the filters gave ten clones that  
 20 hybridized with both probes. These clones were purified by  
 replating and probe hybridization as described above.  
 The cDNA clones were sequenced using an Applied Biosystems  
 373A automated DNA sequencer and Applied Biosystems Taq  
 DyeDeoxy™ Terminator cycle sequencing kits following the  
 25 manufacture's instructions. In some instances, sequences  
 were obtained using [<sup>35</sup>S]dATP (Amersham) and Sequenase™ kits  
 from U.S. Biochemicals following the manufacturer's  
 instructions. Both strands of the cDNA clone 44 were

sequenced by using synthetic oligonucleotides as primers. The sequence of the most 5' 350 nt was determined in seven independent cDNA clones. The resultant clone demonstrated the pattern shown in figure 30 (NDF).

5

EXAMPLE 10Strategies for Detecting Other Possible Splicing Variants

Alignment of the deduced amino acid sequences of the cDNA clones and PCR products of the bovine, and the published human (Fig. 31) and rat sequences show a high level of similarity, indicating that these sequences are derived from homologous genes within the three species. The variable number of messenger RNA transcripts detectable at the cDNA/PCR product level is probably due to extensive tissue-specific splicing. The patterns obtained and shown in Figure 30 suggests that other splicing variants exist. A list of probable splicing variants is indicated in Figure 37. Many of these variants can be obtained by coding segment specific probing of cDNA libraries derived from different tissues and by PCR experiments using primer pairs specific to particular coding segments. Alternatively, the variants can be assembled from specific cDNA clones, PCR products or genomic DNA regions via cutting and splicing techniques known to one skilled in the art. For example, a rare restriction enzyme cutting site in a common coding segment (e.g., A), can be used to connect the FBA amino terminus of GGF2BPP5 to carboxy terminal sequences of GGF2BPP1, GGF2BPP2, GGF2BPP3, or GGF2BPP4. If the presence or the absence of coding segment E and/or G provide benefit for contemplated and stated uses, then these coding segments can be included in expression constructs. These variant

sequences can be expressed in recombinant systems and the recombinant products can be assayed to determine their level of Schwann cell mitogenic activity as well as their ability to bind and activate the p185<sup>erbB2</sup> receptor.

5

EXAMPLE 11Identification of Functional Elements of GGF

The deduced structures of the family of GGF sequences indicate that the longest forms (as represented by GGF2BPP4) encode transmembrane proteins where the  
10 extracellular part contains a domain which resembles epidermal growth factor (see Carpenter and Wahl in Peptide Growth Factors and Their Receptors I pp. 69-133, Springer-Verlag, NY 1991). The positions of the cysteine residues in coding segments C and C/D or C/D' peptide  
15 sequence are conserved with respect to the analogous residues in the epidermal growth factor (EGF) peptide sequence (see Figure 35; SEQ ID Nos. 151-153). This suggests that the extracellular domain functions as receptor recognition and biological activation sites. Several of the  
20 variant forms lack the H, K, and L coding segments and thus may be expressed as secreted, diffusible biologically active proteins. GGF DNA sequences encoding polypeptides which encompass the EGF-like domain (EGFL) can have full biological activity for stimulating glial cell mitogenic  
25 activity.

Membrane bound versions of this protein may induce Schwann cell proliferation if expressed on the surface of neurons during embryogenesis or during nerve regeneration (where the surfaces of neurons are intimately associated  
30 with the surfaces of proliferating Schwann cells).

Secreted (non membrane bound) GGFs may act as classically diffusible factors which can interact with Schwann cells at some distance from their point of secretion. Other forms may be released from intracells by sources via tissue injury and cell disruption. An example of a secreted GGF is the protein encoded by GGF2HBS5 (see example 6); this is the only GGF known which has been found to be directed to the exterior of the cell (example 7). Secretion is probably mediated via an N-terminal hydrophobic sequence found only in region E, which is the N-terminal domain contained within recombinant GGF-II encoded by GGF2HBS5.

Other GGF's appear to be non-secreted (see example 6). These GGFs may be injury response forms which are released as a consequence of tissue damage:

Other regions of the predicted protein structure of GGF-II (encoded by GGF2HBS5) and other proteins containing regions B and A exhibit similarities to the human basement membrane heparan sulfate proteoglycan core protein (ref.). The peptide ADSGEY, which is located next to the second cysteine of the C2 immunoglobulin fold in these GGF's, occurs in nine of twenty-two C-2 repeats found in that basal lamina protein. This evidence strongly suggests that these proteins may associate with matrix proteins such as those associated with neurons and glia, and may suggest a method for sequestration of glial growth factors at target sites.

#### EXAMPLE 12

##### Purification of GGFs from Recombinant Cells



In order to obtain full length or portions of GGFs to assay for biological activity, the proteins can be overproduced using cloned DNA. Several approaches can be used. A recombinant *E. coli* cell containing the sequences described above can be constructed. Expression systems such as pNH8a or pHH16a (Stratagene, Inc.) can be used for this purpose by following manufacturers procedures. Alternatively, these sequences can be inserted in a mammalian expression vector and an overproducing cell line can be constructed. As an example, for this purpose DNA encoding a GGF, clone GGF2BPP5 has been expressed in both COS cells and Chinese hamster ovary cells (see Example 7) (J. Biol. Chem. 263, 3521-3527, (1981)). This vector containing GGF DNA sequences can be transfected into host cells using established procedures.

Transient expression can be examined or G418-resistant clones can be grown in the presence of methotrexate to select for cells that amplify the dhfr gene (contained on the pMSXND vector) and, in the process, co-amplify the adjacent GGF protein encoding sequence. Because CHO cells can be maintained in a totally serum-free, protein-free medium (Hamilton and Ham, In Vitro 13, 537-547 (1977)), the desired protein can be purified from the medium. Western analysis using the antisera produced in Example 9 can be used to detect the presence of the desired protein in the conditioned medium of the overproducing cells.

The desired protein (rGGF-II) was purified from the medium conditioned by transiently expressing cos cells as follows. rGGF-II was harvested from the conditioned medium and partially purified using Cation Exchange Chromatography

(POROS-HS). The column was equilibrated with 33.3 mM MES pH 6.0. Conditioned media was loaded at flow rate of 10 ml/min. The peak containing Schwann cell proliferation activity and immunoreactive (using the polyclonal antisera was against a GGFII peptide described above) was eluted with 50 mM Tris, 1M NaCl pH 8.0. (Figure 50A and 50B respectively).

rGGF-II is also expressed using a stable Chinese Ovary Hamster cell line. rGGF-II from the harvested conditioned media was partially purified using Cation Exchange Chromatograph (POROS-HS). The column was equilibrated with PBS pH 7.4. Conditioned media was loaded at 10 ml/min. The peak containing the Schwann Cell Proliferative activity and immunoreactivity (using GGFII polyclonal antisera) was eluted with 50 mM Hepes, 500 mM NaCl pH 8.0. An additional peak was observed at 50 mM Hepes, 1M NaCl pH 8.0 with both proliferation as well as immunoreactivity (Fig. 51).

rGGF-II can be further purified using Hydrophobic Interaction Chromatography as a high resolution step; Cation exchange/Reserve phase Chromatography (if needed as second high resolution step); A viral inactivation step and a DNA removal step such as Anion exchange chromatography.

Detailed description of procedures used are as follows:

Schwann Cell Proliferation Activity of the recombinant GGF-II peak eluted from the Cation Exchange column was determined as follows: Mitogenic responses of the cultured Schwann cells were measured in the presence of 5 M Forskolin using the peak eluted by 50 mM Tris 1 M NaCl

pH 8.0. The peak was added at 20  $\mu$ l, 10  $\mu$ l (1:10) 10  $\mu$ l and (1:100) 10  $\mu$ l. Incorporation of  $^{125}$ I-Uridine was determined and expressed as (CPM) following an 18-24 hour exposure.

An immunoblot using polyclonal antibody raised  
5 against a peptide of GGF-II was carried out as follows: 10  $\mu$ l of different fractions were ran on 4-12% gradient gels. The gels were transferred on to Nitrocellulose paper, and the nitrocellulose blots were blocked with 5% BSA and probed with GGF-II-specific antibody (1:250 dilution).  $^{125}$ I protein  
10 A (1:500 dilution, Specific Activity = 9.0/Ci/g) was used as the secondary antibody. The immunoblots were exposed to Kodax X-Ray films for 6 hours. The peak fractions eluted with 1 M NaCl showed a broad immunoreactive band at 65-90 Kd which is the expected size range for GGFII and higher  
15 molecular weight glycoforms.

GGF-II purification on cation exchange columns was performed as follows: CHO cell conditioned media expressing rGGFII was loaded on the cation exchange column at 10 ml/min. The column was equilibrated with PBS pH 7.4. The  
20 elution was achieved with 50 mM Hepes 500 mM NaCl pH 8.0 and 50 mM Hepes 1M NaCl pH 8.0 respectively. All fractions were analyzed using the Schwann cell proliferation assay (CPM) described herein. The protein concentration (mg/ml) was determined by the Bradford assay using BSA as the standard.

25 A Western blot using 10  $\mu$ l of each fraction was performed. As indicated in Figure 51A and 51B, immunoreactivity and the Schwann cell activity co-migrates.

The Schwann cell mitogenic assay described herein may be used to assay the expressed product of the full  
30 length clone or any biologically active portions thereof.

The full length clone GGF2BPP5 has been expressed transiently in COS cells. Intracellular extracts of transfected COS cells show biological activity when assayed in the Schwann cell proliferation assay described in Example 1. In addition, the full length close encoding GGF2HBS5 has been expressed transiently in CHO and insect (Example 7) cells. In this case both cell extract and conditioned media show biological activity in the Schwann cell proliferation assay described in Example 1. Any member of the family of splicing variant complementary DNA's derived from the GGF gene (including the Heregulins) can be expressed in this manner and assayed in the Schwann cell proliferation assay by one skilled in the art.

Alternatively, recombinant material may be isolated from other variants according to Wen et al. (Cell 69, 559 (1992)) who expressed the splicing variant Neu differentiation factor (NDF) in COS-7 cells. cDNA clones inserted in the pJT-2 eukaryotic plasmid vector are under the control of the SV40 early promoter, and are 3'-flanked with the SV40 termination and polyadenylation signals. COS-7 cells were transfected with the pJT-2 plasmid DNA by electroporation as follows:  $6 \times 10^6$  cells (in 0.8 ml of DMEM and 10% FBS) were transferred to a 0.4 cm cuvette and mixed with 20  $\mu$ g of plasmid DNA in 10  $\mu$ l of TE solution (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). Electroporation was performed at room temperature at 1600 V and 25  $\mu$ F using a Bio-Rad Gene Pulser apparatus with the pulse controller unit set at 200 ohms. The cells were then diluted into 20 ml of DMEM, 10% FBS and transferred into a T75 flask (Falcon). After 14 hr. of incubation at 37°C, the medium was replaced with DMEM, 1% FBS, and the incubation continued for an

additional 48 hr. Conditioned medium containing recombinant protein which was harvested from the cells demonstrated biological activity in a cell line expressing the receptor for this protein. This cell line (cultured human breast carcinoma cell line AU 565) was treated with recombinant material. The treated cells exhibited a morphology change which is characteristic of the activation of the erbB2 receptor. Conditioned medium of this type also can be tested in the Schwann cell proliferation assay.

10

EXAMPLE 13Purification and Assay of Other Proteins which bind p185<sup>erbB2</sup> ReceptorI. Purification of gp30 and p70

Lupu et al. (Science 249, 1552 (1990)) and Lippman and Lupu (patent application number PCT/US91/03443 (1990)), hereby incorporated by reference, have purified a protein from conditioned media of a human breast cancer cell line MDA-MB-231, as follows.

Conditioned media collections were carried using well-known procedures. The media was concentrated 100-fold in an Amicon ultra-filtration cell (YM5 membrane) (Amicon, Danvers, MA). Once clarified and concentrated, the media were stored at -20°C while consecutive collections were made during the following days. The concentrated media were dialyzed using Spectra/por® 3 tubing (Spectrum Medical Industries, Los Angeles, CA) against 100 volumes of 0.1 M acetic acid over a two day period at 4°C. The material that precipitated during dialysis was removed by centrifugation

at 4000 rpm for 30 min. at 4°C; protease inhibitors were added. The clarified sample was then lyophilized.

Lyophilized conditioned medium was dissolved in 1 M acetic acid to a final concentration of about 25 mg/ml total protein. Insoluble material was removed by centrifugation at 10,000 rpm for 15 minutes. The sample was then loaded onto a Sephadex G-100 column (XK 16, Pharmacia, Piscataway, NJ), was equilibrated and was subjected to elution with 1 M acetic acid at 4°C with an upward flow of 30 ml/hr. 100 ng of protein was processed from 4 ml of 100-fold concentrated medium. Fractions containing 3 ml of eluate were lyophilized and resuspended in 300 µl PBS for assay and served as a source for further purification.

Sephadex G-100 purified material was run on reversed-phase high pressure liquid chromatography (HPLC). The first step involved a steep acetonitrile gradient. Steep acetonitrile gradient and all other HPLC steps were carried out at room temperature after equilibration of the C3-Reversed phase column with 0.05% TFA (Trifluoroacetic acid) in water (HPLC-grade). The samples were loaded and fractions were eluted with a linear gradient (0-45% acetonitrile in 0.05% TFA) at a flow rate of 1 ml/min. over a 30 minute period. Absorbance was monitored at 280 nm. One ml fractions were collected and lyophilized before analysis for EGF receptor-competing activity.

A second HPLC step involved a shallow acetonitrile gradient. The pool of active fractions from the previous HPLC step was rechromatographed over the same column. Elution was performed with a 0-18% acetonitrile gradient in 0.05% TFA over a 5 minute period followed by a linear 18-45% acetonitrile gradient in 0.05% TFA over a 30 minute period.

The flow rate was 1.0 ml/min. and 1 ml fractions were collected. Human TGF $\alpha$ -like factor was eluted at a 30-32% acetonitrile concentration as a single peak detectable by RRA.

5 Lupu et al. (Proc. Natl. Acad. Sci. 89, 2287 (1992)) purified another protein which binds to the p185<sup>erbB2</sup> receptor. This particular protein, p75, was purified from conditioned medium used for the growth of SKBr-3 (a human breast cancer cell line) propagated in improved Eagle's  
10 medium (IMEM: GIBCO) supplemented with 10% fetal bovine serum (GIBCO). Protein p75 was purified from concentrated (100X) conditioned medium using a p185<sup>erbB2</sup> affinity column. The 94 Kilodalton extracellular domain of p185<sup>erbB2</sup> (which binds p75) was produced via recombinant expression and was  
15 coupled to a polyacrylamide hydrazido-Sepharose affinity chromatography matrix. Following coupling the matrix was washed extensively with ice cold 1.0 M HCl and the beads were activated with 0.5 M NaNO<sub>2</sub>. The temperature was maintained at 0°C for 20 minutes and this was followed by  
20 filtration and washing with ice cold 0.1 M HCl. 500 ml of concentrated conditioned medium was run through the beads by gravity. The column was washed and eluted stepwise with 1.0 M citric acid at pH values from 4.0 to 2.0 (to allow dissociation of the erbB2 and p75). All fractions were  
25 desalted on Pharmacia PD10 columns. Purification yielded a homogeneous polypeptide of 75kDa at 3.0-3.5 elution pH (confirmed by analysis on SDS/PAGE by silver staining).

## II. Binding of gp30 to p185<sup>erbB2</sup>

The purified gp30 protein was tested in an assay to  
30 determine if it bound to p185<sup>erbB2</sup>. A competition assay with

- a monoclonal antibody against p185<sup>erbB2</sup>. The gp30 protein displaced antibody binding to p185<sup>erbB2</sup> in SK-BR-3 and MDA-MB-453 cells (human breast carcinoma cell lines expressing the p185<sup>erbB2</sup> receptor). Schwann cell
- 5 proliferation activity of gp30 can also be demonstrated by treating Schwann cell cultures with purified gp30 using the assay procedure described in Examples 1-3.

### III. Binding of p75 to p185<sup>erbB2</sup>

- To assess whether the 75-kDa polypeptide (p75)
- 10 obtained from SKBr-3 conditioned medium was indeed a ligand for the erbB2 oncoprotein in SKBr-3 cells, a competition assay as described above for gp30 was used. It was found that the p75 exhibited binding activity, whereas material from other chromatography fractions did not show such
- 15 activity (data not shown). The flow-through material showed some binding activity. This might be due to the presence of shed erbB2 ECD.

### IV. Other p185<sup>erbB2</sup> ligands

- Peles et al. (Cell 69, 205 (1992)) have also
- 20 purified a 185<sup>erbB2</sup> stimulating ligand from rat cells, (NDF, see Example 8 for method). Holmes et al. (Science 256, 1205 (1992)) have purified Heregulin  $\alpha$  from human cells which binds and stimulates 185<sup>erbB2</sup> (see example 6). Tarakovsky et al. Oncogene 6:218 (1991) have demonstrated binding of a 25
- 25 kD polypeptide isolated from activated macrophages to the Neu receptor, a p185<sup>erbB2</sup> homology, herein incorporated by reference.

### VI. NDF Isolation



Yarden and Peles (Biochemistry 30, 3543 (1991)) have identified a 35 kilodalton glycoprotein which will stimulate the 185<sup>erbB2</sup> receptor. The protein was identified in conditioned medium according to the following procedure.

- 5 Rat I-EJ cells were grown to confluence in 175-cm<sup>2</sup> flasks (Falcon). Monolayers were washed with PBS and left in serum-free medium for 10-16 h. The medium was discarded and replaced by fresh serum-free medium that was collected after 3 days in culture. The conditioned medium was cleared by
- 10 low-speed centrifugation and concentrated 100-fold in an Amicon ultrafiltration cell with a YM2 membrane (molecular weight cutoff of 2000). Biochemical analyses of the *neu* stimulatory activity in conditioned medium indicate that the ligand is a 35-kD glycoprotein that it is heat stable but
- 15 sensitive to reduction. The factor is precipitable by either high salt concentrations or acidic alcohol. Partial purification of the molecule by selective precipitation, heparin-agarose chromatography, and gel filtration in dilute acid resulted in an active ligand, which is capable of
- 20 stimulating the protooncogenic receptor but is ineffective on the oncogenic *neu* protein, which is constitutively active. The purified fraction, however, retained the ability to stimulate also the related receptor for EGF, suggesting that these two receptors are functionally coupled
- 25 through a bidirectional mechanism. Alternatively, the presumed ligand interacts simultaneously with both receptors. The presented biochemical characteristic of the factor may be used to enable a completely purified factor with which to explore these possibilities.

- 30 In other publications, Davis et al. (Biochem. Biophys. Res. Commun. 179, 1536 (1991), Proc. Natl. Acad.

Sci. 88, 8582 (1991) and Greene et al., PCT patent application PCT/US91/02331 (1990)) describe the purification of a protein from conditioned medium of a human T-cell (ATL-2) cell line.

5 ATL-2 cell line is an IL-2-independent HTLV-1 (+) T cell line. Mycoplasma-free ATL-2 cells were maintained in RPMI 1640 medium containing 10% FCS as the culture medium (10% FCS-RPMI 1640) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

10 For purification of the proteinaceous substance, ATL-2 cells were washed twice in 1 x PBS and cultured at 3 x 10<sup>5</sup> ml in serum-free RPMI 1640 medium/2 mM L-glutamine for seventy-two hours followed by pelleting of the cells. The culture supernatant so produced is termed "conditioned medium" (C.M.).

15 C.M. was concentrated 100 fold, from 1 liter to 10 ml, using a YM-2 Diaflo membrane (Amicon, Boston, MA) with a 1000d cutoff. For use in some assays, concentrated C.M. containing components greater than 1000 MW were rediluted to 20 original volume with RPMI medium. Gel electrophoresis using a polyacrylamide gradient gel (Integrated Separation Systems, Hyde Park, MD or Phorecast System by Amersham, Arlington Heights, IL) followed by silver staining of some of this two column purified material from the one liter 25 preparation revealed at least four to five bands of which the 10kD and 20kD bands were unique to this material. Passed C.M. containing components less than 1000 NW were used without dilution.

30 Concentrated conditioned medium was filter sterilized with a .45μ uniflo filter (Schleicher and Schuell, Keene, NH) and then further purified by application

to a DEAE-SW anion exchange column (Waters, Inc., Milford, MA) which had been preequilibrated with 10mM Tris-Cl, pH 8.1 Concentrated C.M. proteins representing one liter of original ATL-2 conditioned medium per HPLC run were absorbed  
5 to the column and then eluted with a linear gradient of 0mM to 40mM NaCl at a flow rate of 4 ml/min. Fractions were assayed using an in vitro immune complex kinase assay with 10% of the appropriate DEAE fraction (1 column purified material) or 1% of the appropriate C18 fractions (two column  
10 purified material). The activity which increased the tyrosine kinase activity of p185c-neu in a dose-dependent manner using the in vitro immune complex kinase assay was eluted as one dominant peak across 4 to 5 fractions (36-40) around 220 to 240 mM of NaCl. After HPLC-DEAE purification,  
15 the proteins in the active fractions were concentrated and pooled, concentrated and subjected to C18 (million matrix) reverse phase chromatography (Waters, Inc., Milford, MA) (referred to as the C18+1 step or two column purified material). Elution was performed under a linear gradient of  
20 2-propanol against 0.1% TFA. All the fractions were dialyzed against RPMI 1640 medium to remove the 2-propanol and assayed using the in vitro immune complex kinase assay, described below, and a 1% concentration of the appropriate fraction. The activity increasing the tyrosine kinase  
25 activity of p185c-neu was eluted in two peaks. One eluted in fraction 11-13, while a second, slightly less active peak of activity eluted in fractions 20-23. These two peaks correspond to around 5 to 7% of isopropanol and 11 to 14% isopropanol respectively. C18#1 generated fractions 11-13  
30 were used in the characterization studies. Active fractions

obtained from the second chromatographic step were pooled, and designated as the proteinaceous substance sample.

A twenty liter preparation employed the same purification strategy. The DEAE active fractions 35-41 were  
5 pooled and subjected to C18 chromatography as discussed above. C18#1 fractions 11-13 and 21-24 both had dose-dependent activity. The pool of fractions 11-13 was subjected to an additional C18 chromatographic step (referred to as C18#2 or three column purified material).  
10 Again, fractions 11-13 and 21-24 had activity. The dose response of fraction 23 as determined by in vitro immune complex kinase assay as described in Example 8 may be obtained upon addition of 0.005% by volume fraction 23 and 0.05% by volume fraction 23. This represents the greatest  
15 purity achieved.

Molecular weight ranges were determined based on gel filtration chromatography and ultrafiltration membrane analysis. Near equal amounts of tyrosine kinase activity were retained and passed by a 10,000 molecular weight cut  
20 off filter. Almost all activity was passed by a 30,000 molecular weight cut off filter. Molecular weight ranges for active chromatographic fractions were determined by comparing fractions containing dose-dependent neu-activating activity to the elution profiles of a set of protein  
25 molecular weight standards (Sigma Chemical Co., St. Louis, MO) generated using the same running conditions. A low molecular weight region of activity was identified between 7,000 and 14,000 daltons. A second range of activity ranged from about 14,000 to about 24,000 daltons.

30 After gel electrophoresis using a polyacrylamide gradient gel (Integrated Separation Systems, Hyde Park, MD

or Phorecase System by Amersham, Arlington Heights, IL), silver staining of the three-column purified material (c18#2) was done with a commercially available silver staining kit (BioRad, Rockville Centre, NY). Fraction 21, 22, 23, and 24 from c18#2 purification of the twenty liter preparation were run with markers. Fractions 22 and 23 showed the most potent dose response in the 185<sup>erbB2</sup> (neu) kinase assay (see below). The fact that selected molecular weight fractions interact with 185<sup>erbB2</sup> was demonstrated with an immune complex kinase assay.

Huang et al. (1992, J. Biol. Chem. 257:11508-11512), hereby incorporated by reference, have isolated an additional neu/erb B2 ligand growth factor from bovine kidney. The 25 kD polypeptide factor was isolated by a procedure of column fractionation, followed by sequential column chromatography on DEAE/cellulose (DE52), Sulfadex (sulfated Sephadex G-50), heparin-Sepharose 4B, and Superdex 75 (fast protein liquid chromatography). The factor, NEL-GF, stimulates tyrosine-specific autophosphorylation of the neu/erb B2 gene product.

VII. Immune complex assay NDF for ligand binding to p185<sup>erbB2</sup>: This assay reflects the differences in the autophosphorylation activity of immunoprecipitated p185 driven by pre-incubation of PN-NR6 cell lysate with varying amounts of ATL-2 conditioned medium (C.H.) or proteinaceous substance and is referred to hereinafter as neu-activating activity.

Cell lines used in the immune complex kinase assay were obtained, prepared and cultured according to the methods disclosed in Kokai et al., Cell 55, 287-292 (July

28, 1989) the disclosures of which are hereby incorporated by reference as if fully set forth herein, and U.S. application serial number 386,820 filed July 27, 1989 in the name of Mark I. Green entitled "Methods of Treating  
5 Cancerous Cells with Anti-Receptor Antibodies", the disclosures of which are hereby incorporated by reference as if fully set forth herein.

Cell lines were all maintained in DMEM medium containing 5% FCS as the culture medium (5% FCS-DMEM) at  
10 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

Dense cultures of cells in 150 mm dishes were washed twice with cold PBS, scraped into 10 ml of freeze-thaw buffer (150 mM NaCl, 1 mM MgCl<sub>2</sub>, 20 mM Hepes, pH 7.2, 10% Glycerol, 1 mM EDTA, 1% Aprotinin), and centrifuged (600 x  
15 6, 10 minutes). Cell pellets were resuspended in 1 ml Lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 3% Brij 35, 1 mM EDTA, 1.5 mM MgCl<sub>2</sub>, 1% Aprotinin, 1 mM EGTA, 20 μM Na<sub>2</sub>VO<sub>4</sub>, 10% Glycerol) and rotated for thirty minutes at 4°C. All chemicals were from Sigma Chemical Co., St. Louis, Mo,  
20 unless otherwise indicated. The insoluble materials were removed by centrifugation at 40,000 x g for thirty minutes. The clear supernatant which was subsequently used is designated as cell lysate.

The cell lysates were incubated for fifteen minutes  
25 with 50 μl of 50% (volume/volume) Protein A-sepharose (Sigma Chemical Co., St. Louis, Missouri), and centrifugated for two minutes to preclear the lysates. 50 μl aliquots of precleared cell lysate were incubated on ice for fifteen minutes with conditioned medium, proteinaceous substance, or  
30 other factors as specified, in a final volume of 1 ml with lysis buffer. The sample was then incubated with 5 μg of

7.16.4 monoclonal antibody, which recognizes the extracellular domain of the p185neu and p185c-neu, or other appropriate antibodies, for twenty minutes on ice, followed by a twenty minute incubation with 50  $\mu$ l of 50% (vol/vol) protein A-Sepharose with rotation at 4°C. Immune complexes were collected by centrifugation, washed four times with 500  $\mu$ l of washing buffer (50 mM Hepes, pH 7.5, 0.1% Brij 35, 150 mM NaCl, 2 mM EDTA, 1% Aprontinin, 30  $\mu$ M Na<sub>2</sub>VO<sub>4</sub>), then twice with reaction buffer (20 mM Hepes (pH 7.4), 3 mM MnCl<sub>2</sub> and 0.1% Brij 35, 30  $\mu$ M Na<sub>2</sub>VO<sub>4</sub>). Pellets were resuspended in 50  $\mu$ l of reaction buffer and (Gamma-<sup>32</sup>P]-ATP (Amersham, Arlington Heights, IL) was added giving a final concentration of 0.2  $\mu$ M. The samples were incubated at 27°C for twenty minutes or at 4°C for 25 minutes with purer samples. The reactions were terminated by addition of 3 x SDS sample buffer containing 2 mM ATP and 2 mM EDTA and then incubating them at 100°C for five minutes. The samples were then subjected to SDS-PAGE analysis on 10% acrylamide gels. Gels were stained, dried, and exposed to Kodak XAR or XRP film with intensifying screens.

#### VIII. Purification of acetylcholine receptor inducing activity (ARIA)

ARIA, a 42 kD protein which stimulates acetylcholine receptor synthesis, has been isolated in the laboratory of Gerald Fischbach (Falls et al., Cell 72:801-815 (1993)). ARIA induces tyrosine phosphorylation of a 185 Kda muscle transmembrane protein which resembles p185<sup>erbB2</sup>, and stimulates acetylcholine receptor synthesis in cultured embryonic myotubes. Sequence analysis of cDNA clones which encode ARIA shows that ARIA is a member of the GGF/erbB2

ligand group of proteins, and this is potentially useful in the glial cell mitogenesis stimulation and other applications of, e.g., GGF2 described herein.

#### EXAMPLE 14

##### 5 Protein tyrosine phosphorylation mediated by GGF in Schwann cells

Rat Schwann cells, following treatment with sufficient levels of Glial Growth Factor to induce proliferation, show stimulation of protein tyrosine phosphorylation (figure 36). Varying amounts of partially purified GGF were applied to a primary culture of rat Schwann cells according to the procedure outlined in Example 3. Schwann cells were grown in DMEM/10% fetal calf serum/5  $\mu$ M forskolin/0.5  $\mu$ g per mL GGF-CM (0.5mL per well) in poly D-lysine coated 24 well plates. When confluent, the cells were fed with DMEM/10% fetal calf serum at 0.5mL per well and left in the incubator overnight to quiesce. The following day, the cells were fed with 0.2mL of DMEM/10% fetal calf serum and left in the incubator for 1 hour. Test samples were then added directly to the medium at different concentrations and for different lengths of time as required. The cells were then lysed in boiling lysis buffer (sodium phosphate, 5mM, pH 6.8; SDS, 2%,  $\beta$ -mercapteothanol, 5%; dithiothreitol, 0.1M; glycerol, 10%; Bromophenol Blue, 0.4%; sodium vanadate, 10mM), incubated in a boiling water bath for 10 minutes and then either analyzed directly or frozen at -70°C. Samples were analyzed by running on 7.5% SDS-PAGE gels and then electroblotting onto nitrocellulose using standard procedures as described by Towbin et al. (1979) Proc. Natl. Acad. Sci. USA 76:4350-4354. The blotted



nitrocellulose was probed with antiphosphotyrosine antibodies using standard methods as described in Kamps and Selton (1988) Oncogene 2:305-315. The probed blots were exposed to autoradiography film overnight and developed using a standard laboratory processor. Densitometric measurements were carried out using an Ultrascan XL enhanced laser densitometer (LKB). Molecular weight assignments were made relative to prestained high molecular weight standards (Sigma). The dose responses of protein phosphorylation and Schwann cell proliferation are very similar (figure 36). The molecular weight of the phosphorylated band is very close to the molecular weight of p185<sup>erbB2</sup>. Similar results were obtained when Schwann cells were treated with conditioned media prepared from COS cells translates with the GGF2HBS5 clone. These results correlate well with the expected interaction of the GGFs with and activation of 185<sup>erbB2</sup>.

This experiment has been repeated with recombinant GGF-II. Conditioned medium derived from a CHO cell line stably transformed with the GGF-II clone (GGF2HBS5) stimulates protein tyrosine phosphorylation using the assay described above. Mock transfected CHO cells fail to stimulate this activity (Fig. 52).

#### EXAMPLE 15

Assay for Schwann cell Proliferation by Protein Factor from the MDA-MB-231 cell line.

Schwann cell proliferation is mediated by conditioned medium derived from the human breast cancer cell line MDA-MB-231. On day 1 of the assay, 10<sup>4</sup> primary rat

Schwann cells were plated in 100  $\mu$ l of Dulbecco's Modified Eagle's medium supplemented with 5% fetal bovine plasma per well in a 96 well microtiter plate. On day 2 of the assay, 10  $\mu$ l of conditioned medium (from the human breast cancer cell line MDA-MB-231, cultured as described in Example 6) was added to each well of the microtiter plate. One day 6, the number of Schwann cells per plate was determined using an acid phosphatase assay (according to the procedure of Connolly et al. Anal. Biochem. 152: 136 (1986)). The plate was washed with 100  $\mu$ l of phosphate buffered saline (PBS) and 100  $\mu$ l of reaction buffer (0.1M sodium acetate, (pH 5.5)), 0.1% Triton X-100, and 10 mM p-nitrophenyl phosphate) was added per well. The plate was incubated at 37°C for two hours and the reaction was stopped by the addition of 10  $\mu$ l of 1N NaOH. The optical density of each sample was read in a spectrophotometer at 410 nm. A 38% stimulation of cell number over Schwann cells treated with conditioned medium from a control cell line (HS-294T, a non-producer of erbB-2 ligand) was observed. This result shows that a protein secreted by the MDA-MB-231 cell line (which secretes a p185<sup>erbB2</sup> binding activity) stimulates Schwann cell proliferation.

#### EXAMPLE 16

##### N-glycosylation of GGF

The protein sequence predicted from the cDNA sequence of GGF-II candidate clones GGF2BPP1,2 and 3 contains a number of consensus N-glycosylation motifs. A gap in the GGFI102 peptide sequence coincides with the asparagine residue in one of these motifs, indicating that carbohydrate is probably bound at this site.

N-glycosylation of the GGFs was studied by observing mobility changes on SDS-PAGE after incubation with N-glycanase, an enzyme that cleaves the covalent linkages between carbohydrate and asparagine residues in proteins.

- 5 N-Glycanase treatment of GGF-II yielded a major band of MW 40-42 kDa and a minor band at 45-48 kDa. Activity elution experiments under non-reducing conditions showed a single active deglycosylated species at ca 45-50 kDa.

- 10 Activity elution experiments with GGF-I also demonstrate an increase in electrophoretic mobility when treated with N-Glycanase, giving an active species of MW 26-28 kDa. Silver staining confirmed that there is a mobility shift, although no N-deglycosylated band could be assigned because of background staining in the sample used.

EXAMPLE 17

Further tests were carried out to determine the mature GGF2 protein once the protein is expressed and secreted from transfected cells.

5           The cDNA encoding human GGF2 was cloned into an amplified vector pcdhfrpolyA and transfected into CHO-DG44 cells for stable expression. rhGGF2 is secreted into the conditioned media. The ability of the recombinant GGF2 to be secreted is mediated through the N-terminal hydrophobic stretch, i.e., signal sequence. According to the signal  
10           hypotheses, a signal sequence, once having initiated the export of a growing protein chain across the rough endoplasmic reticulum, is cleaved from the mature protein at a specific site. N-terminal analysis of the expressed and  
15           purified rhGGF2 indicates that the site of cleavage is between A<sub>50</sub> and G<sub>51</sub>. The first 50 amino acid residues are cleaved from the mature protein, thus rhGGF2 consists of 373 amino acids. The amino acid sequence of the cDNA encoding hGGF2 can be found in Figure 55.

20           The first fifteen amino acid residues at the N-terminal of the protein is confirmed by N-terminal sequence analysis as follows in Table 1.

Table 1 - N-terminal sequence analysis of rhGGF2

25	Cycle #	Primary Sequence	pMoles
	-----		
	1	Gly(G)	210.6
	2	Asn(N)	163

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	3	Glu(E)	149
	4	Ala(A)	220
	5	Ala(A)	180
5	6	Pro(P)	173
	7	Ala(A)	177
	8	Gly(G)	154.9
	9	Ala(A)	162.4
	10	Ser(S)	65.4
	11	Val(V)	132.7
10	12	Val(V) (Cys)*	11.7
	13	Tyr(Y)	112.7
	14	Ser(S)	47.6
	15	Ser(S)	27.1

The N-terminal sequence analysis is performed by  
 15 Edman Degradation Process. The \*Cys residues are destroyed  
 by the Edman Degradation Process and cannot be detected.

#### Deposit

Nucleic acid encoding GGF-II (cDNA, GGF2HBS5)  
 protein (Example 6) in a plasmid pBluescript 5k, under the  
 20 control of the T7 promoter, was deposited in the American  
 Type Culture Collection, Rockville, Maryland, on September  
 2, 1992, and given ATCC Accession No. 75298. Applicant  
 acknowledges its responsibility to replace this plasmid  
 should it become non-viable before the end of the term of a  
 25 patent issued hereon, and its responsibility to notify the  
 ATCC of the issuance of such a patent, at which time the  
 deposit will be made available to the public. Prior to that

time the deposit will be made available to the Commissioner  
of Patents under the terms of 37 CFR §1.14 and 35 USC §112.

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Applicant or agent's file reference number	LUD 5250.5 PCT	International application No.	PCT/US95/06846
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13b4s)

A. The indications made below relate to the microorganism referred to in the description on page <u>107</u> , line <u>17-22</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet: <input type="checkbox"/>	
Name of depository institution American Type Culture Collection	
Address of depository institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States	
Date of deposit 2 September 1992	Accession Number 75298
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet: <input type="checkbox"/>	
nucleic acid encoding GGF2HBS5 protein	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (If the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable) The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., 'Accession Number of Deposit')	
For receiving Office use only <input checked="" type="checkbox"/> This sheet was received with the international application Authorized officer Doris L. Brock <i>DLB</i> PCT International Division	For International Bureau use only <input type="checkbox"/> This sheet was received by the International Bureau on Authorized officer

Form PCT/IV/13 (July 1992)

SUBSTITUTE SHEET (RULE 26)

What is claimed is:

1. A basic polypeptide factor having Schwann cell mitogenic activity, wherein said polypeptide factor lacks an N-terminal signal sequence.

5           2. The basic polypeptide factor of claim 1, wherein said polypeptide factor has an amino acid sequence defined as amino acid position 51 to amino acid position 422 in Figure 55 (SEQ. ID No. 179).

          3. Isolated DNA sequence encoding a polypeptide  
10 factor having Schwann cell mitogenic activity, wherein said DNA sequence lacks an N-terminal signal sequence.

          4. An isolated DNA sequence encoding the polypeptide factor of claim 2.

          5. A method for stimulating mitogenesis of a glial  
15 cell, said method comprising contacting said glial cell with an effective amount of said polypeptide of claim 1.

          6. A method for the prophylaxis or treatment of a pathophysiological condition of the nervous system in a



mammal in which said condition involves a cell type which is sensitive or responsive to the polypeptide of claim 1, said method comprising administering to said mammal an effective amount of said polypeptide.

5           7. A polypeptide having a glial cell mitogenic activity wherein said polypeptide encoded by the DNA sequence of claim 3, said polypeptide obtained by a method comprising cultivating modified host cells under conditions permitting expression of said DNA sequence.

10           8. Method for identifying the presence of a receptor for the polypeptide of claim 1, in a sample comprising contacting said sample to said polypeptide and determining binding therebetween, wherein said binding is indicative of the presence of said receptor.

15           9. A method for the prophylaxis or treatment of a glial tumor in a patient, said method comprising administering to said patient an effective amount of a substance which inhibits the binding of a polypeptide of claim 1 to a receptor therefor.

10. A pharmaceutical or veterinary formulation comprising a polypeptide of claim 1, formulated for pharmaceutical or veterinary use, respectively, together with an acceptable diluent, carrier or excipient and/or in  
5 unit dosage form.

11. A method for the treatment of a condition which involves peripheral nerve damage in a mammal, said method comprising contacting said peripheral nerves with an effective amount of a polypeptide of claim 1.

10 12. A method for the prophylaxis or treatment of a condition in a mammal wherein said condition involves demyelination or damage or loss of Schwann cells, said method comprising contacting said Schwann an effective amount of a polypeptide of claim 1.

15 13. The method of claim 12 wherein said condition is a neuropathy of sensory or motor nerve fibers.

14. A method for the prophylaxis or treatment of a neurodegenerative disorder in a mammal, said method comprising contacting glial cells in a mammal with an  
20 effective amount of a polypeptide of claim 1.

15. A method for inducing neural regeneration and/or repair in a mammal, said method comprising contacting glial cells in a mammal with an effective amount of a polypeptide of claim 1.

5 16. A method of inducing fibroblast proliferation, said method comprising contacting said fibroblasts with a polypeptide, of claim 1.

17. A method of wound repair in mammals, said method comprising contacting said wound with a polypeptide  
10 of claim 1.

18. A method of making a medicament comprising admixing a polypeptide of claim 1, with a pharmaceutically acceptable carrier.

19. A method for producing an antibody, said method  
15 comprising immunizing a mammal with a polypeptide of claim 1.

20. A method for detecting a receptor which is capable of binding to a polypeptide of claim 1, said method

comprising carrying out affinity isolation on said sample using a said peptide as the affinity ligand.

21. A method for the prophylaxis or treatment of a glial tumor in a patient, said method comprising  
5 administering to said patient an effective amount of a substance which inhibits the binding of a polypeptide of claim 1, to a receptor therefor.

22. A method of investigating, isolating or preparing a glial cell mitogen or gene sequence encoding  
10 said glial cell mitogen, said method comprising contacting tissue preparations or samples with an antibody, said antibody prepared as defined in claim 19.

23. A method for isolating a nucleic acid sequence coding for a molecule having glial cell mitogenic activity,  
15 said method comprising contacting a cell containing sample with a glial cell mitogen specific antibody to determine expression of said mitogen in said sample and isolating said nucleic acid sequence from the cells exhibiting said expression.

24. A method for inducing myelination of a neural cell by a Schwann cell, said method comprising contacting said Schwann cell with a polypeptide of claim 1.

25. A method for inducing acetylcholine receptor synthesis in a cell, said method comprising contacting of said cell with a polypeptide of claim 1.

26. An antibody to a polypeptide as defined in claim 1.

27. A method of purifying a protein with glial cell mitogenic activity, said method comprising contacting a cell extract with an antibody of claim 26.

28. A method of treating a mammal suffering from a disease of glial cell proliferation, said method comprising administering to said mammal an antibody of claim 26.

29. A vector comprising the DNA sequence of claim 3.

30. A host cell containing the isolated DNA of claim 3.

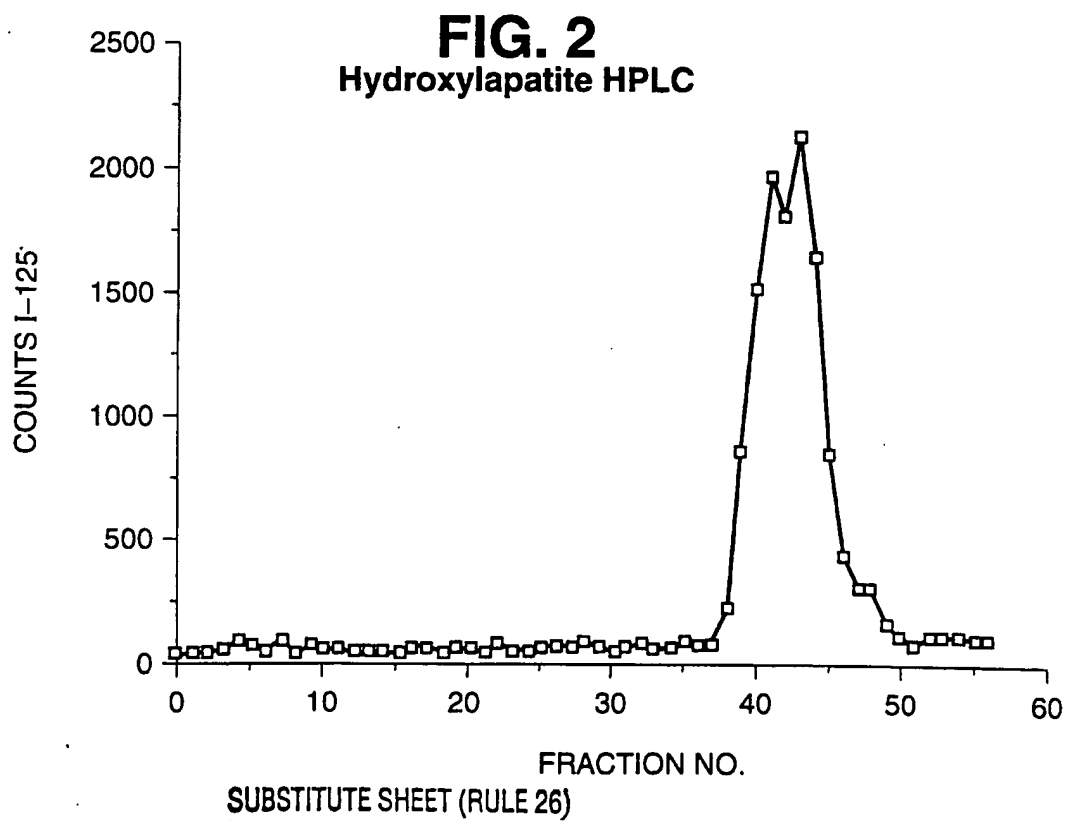
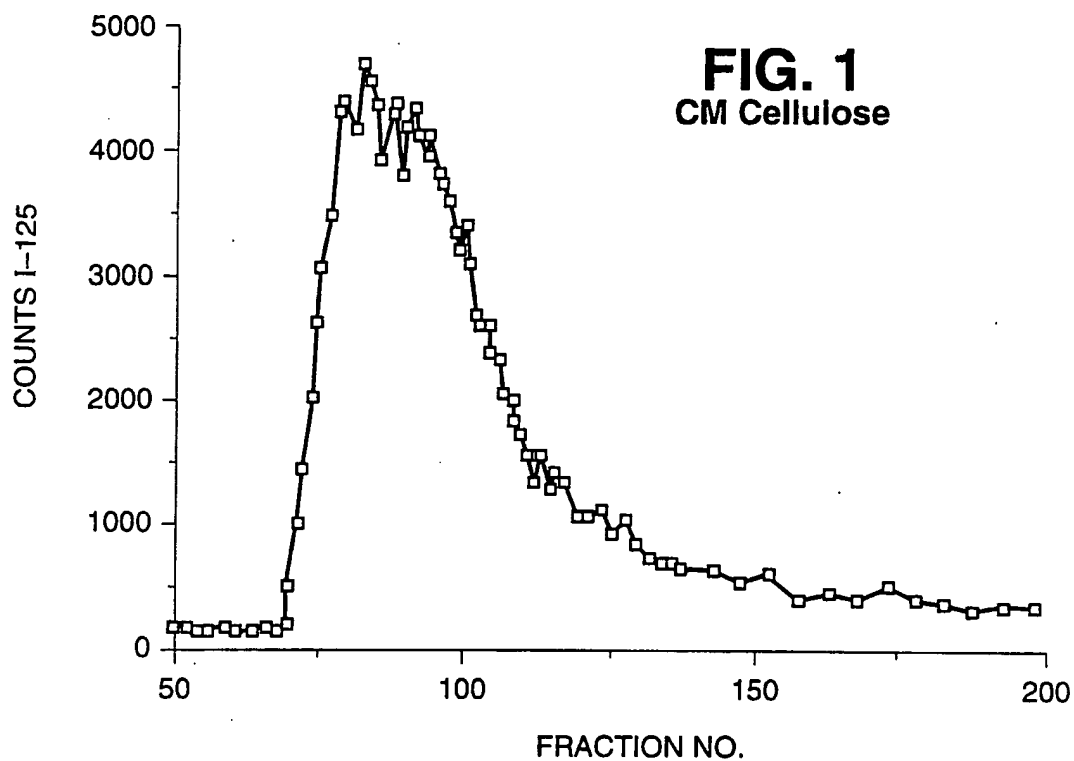
31. A method for the preparation of a glial cell mitogenic factor, said method comprising cultivating the host cell of claim 30, under conditions permitting expression of said DNA sequence.

5           32. A polypeptide of claim 1 for use as a glial cell mitogen.

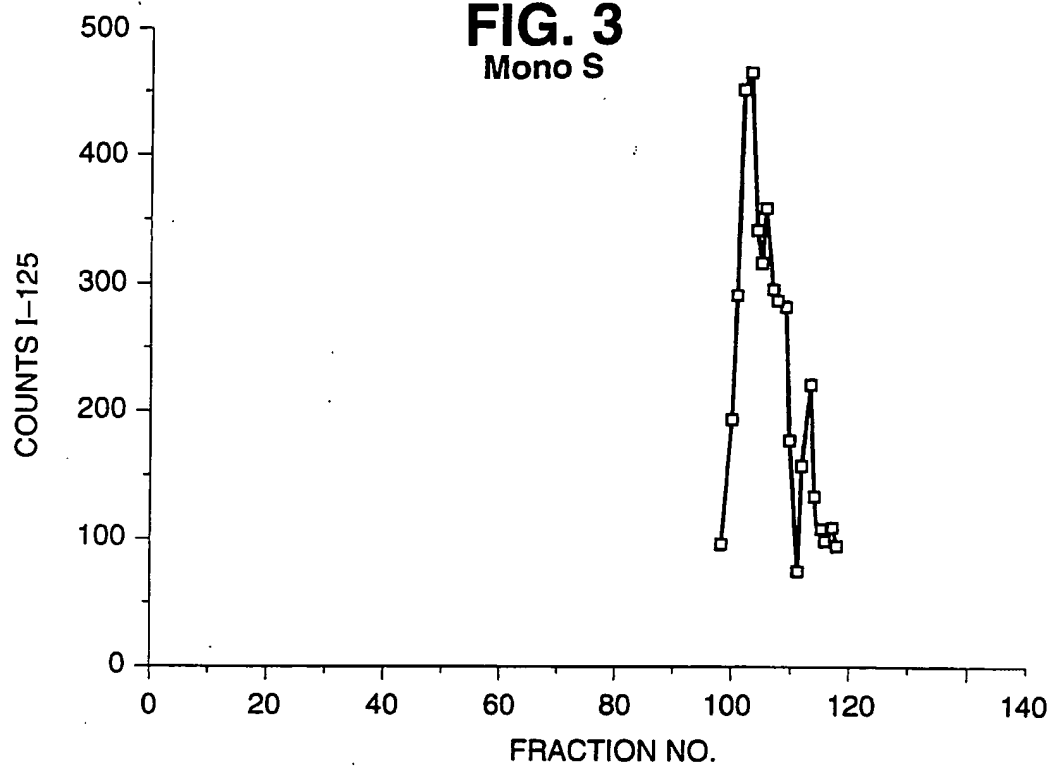
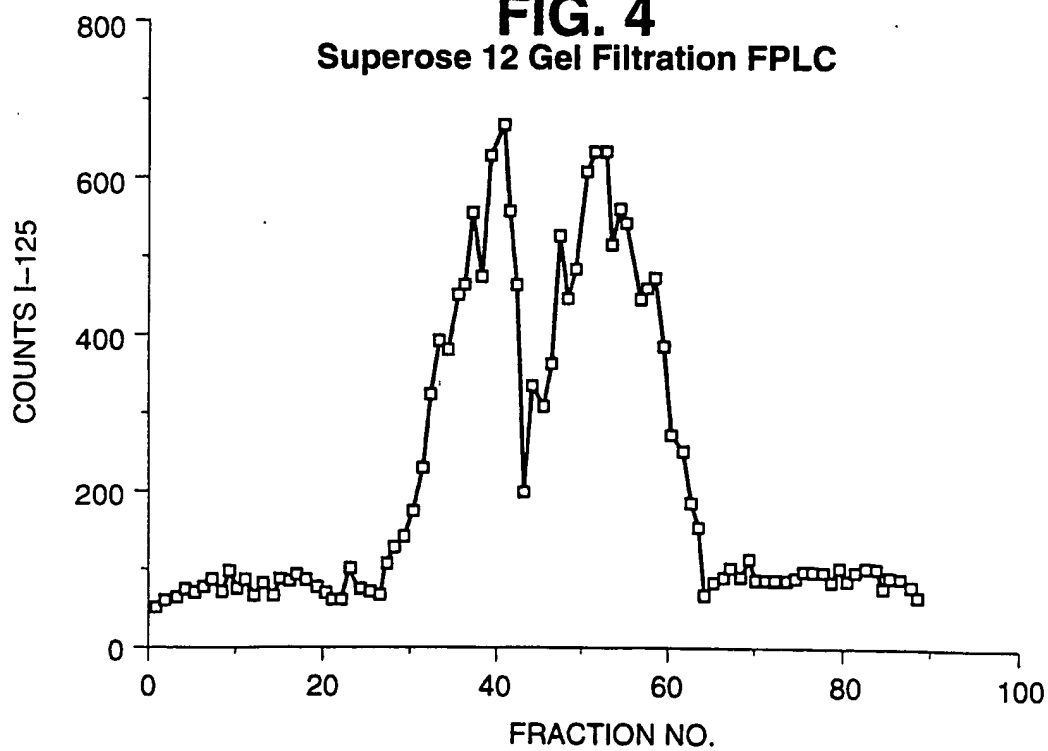
33. A method for the prophylaxis or treatment of multiple sclerosis in a patient, said method comprising administering to said patient an effective amount of a  
10 substance which inhibits the binding of a polypeptide of claim 1 to a receptor therefor.

34. A polypeptide which is a glial cell mitogen, said polypeptide being encoded by a DNA sequence as defined in claim 3, said polypeptide obtained by a method comprising  
15 for the preparation of a glial cell mitogenic factor, said method cultivating modified host cells under conditions permitting expression of said DNA sequence.

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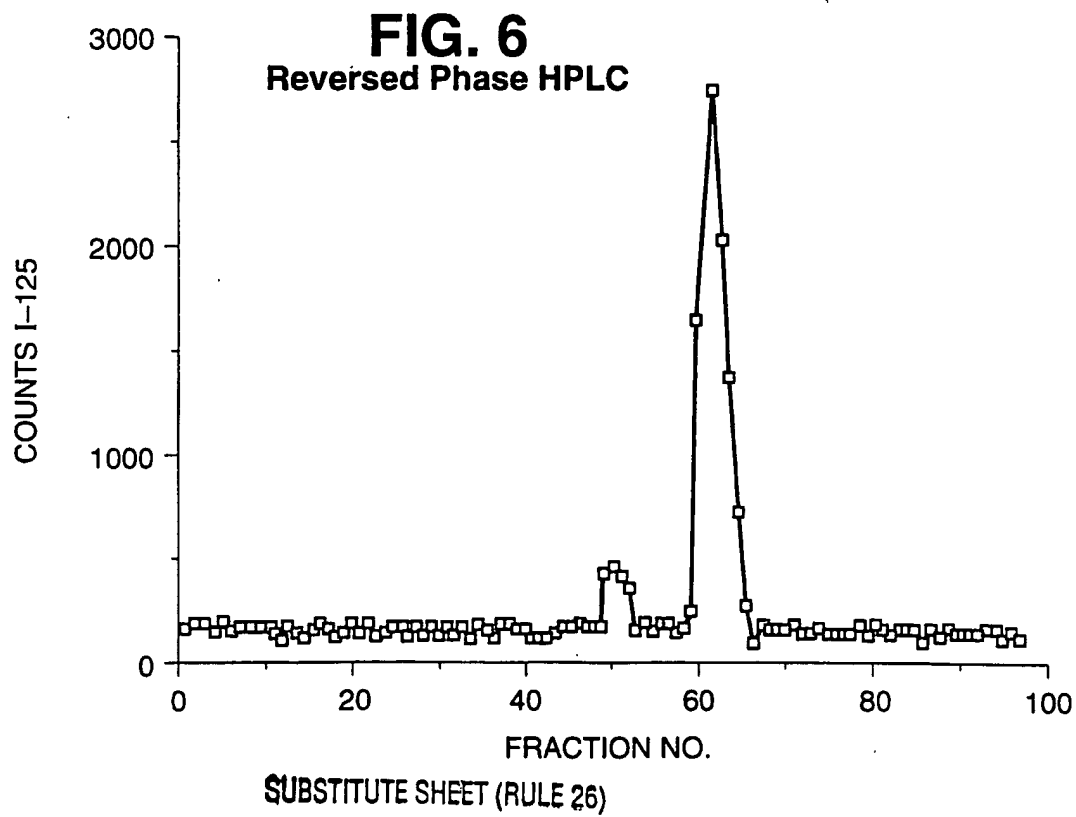
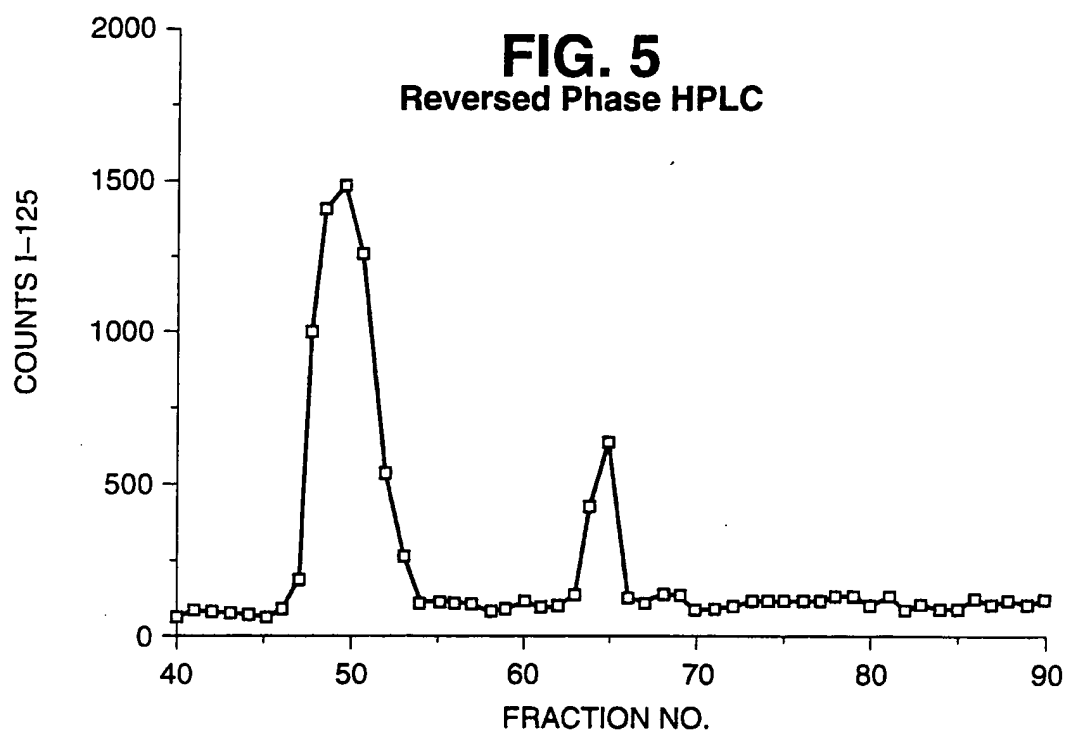


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**FIG. 3**  
Mono S**FIG. 4**  
Superose 12 Gel Filtration FPLC

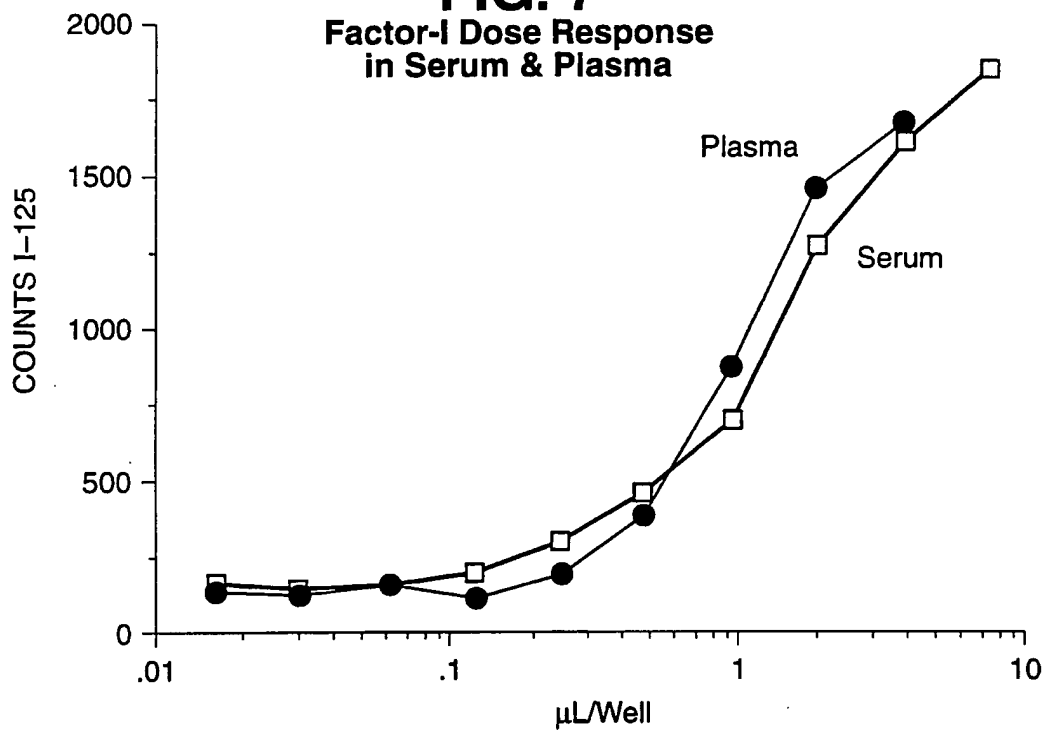


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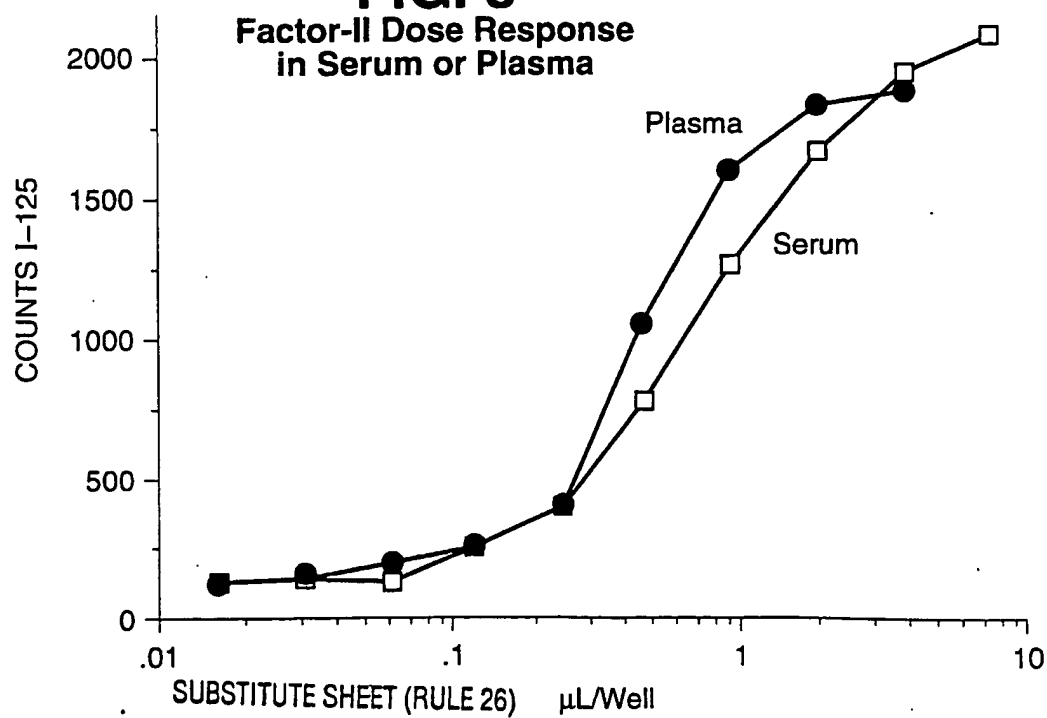


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**FIG. 7**  
Factor-I Dose Response  
in Serum & Plasma



**FIG. 8**  
Factor-II Dose Response  
in Serum or Plasma



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## FIG. 9

GGF-I	01	N-terminus F K G D A H T E		(SEQ ID NO: 1)
GGF-I	02	Trypsin peptides K/R A S L A D E Y E Y M X K *		(SEQ ID NO: 2)
GGF-I	03	K/R T E T S S S G L X L K *		(SEQ ID NO: 3)
GGF-I	04	K/R K L G E M W A E		(SEQ ID NO: 4)
GGF-I	05	K/R L G E K R A		(SEQ ID NO: 5)
GGF-I	06	K/R I K S E H A G L S I G D T A K *	HMG-1	(SEQ ID NO: 6)
GGF-I	07	K/R A S L A D E Y E Y M R K *	HMG-1?	(SEQ ID NO: 7)
GGF-I	08	K/R I K G E H P G L S I G D V A K *	HMG-2	(SEQ ID NO: 8)
GGF-I	09	K/R M S E Y A F F V Q T X R *		(SEQ ID NO: 9)
GGF-I	10	K/R S E H P G L S I G D T A K *	HMG-1	(SEQ ID NO: 10)
GGF-I	11	K/R A G Y F A E X A R *	HMG-2	(SEQ ID NO: 11)
GGF-I	12	K/R K L E F L X A K *	HMG-1	(SEQ ID NO: 12)
GGF-I	13	K/R T E M A S E Q G A		(SEQ ID NO: 13)
GGF-I	14	K/R A K E A L A A L K *		(SEQ ID NO: 14)
GGF-I	15	K/R F V L Q A K K *		(SEQ ID NO: 15)
GGF-I	16	K/R L G E M W	HMG-1	(SEQ ID NO: 16)
GGF-I	17	Protease V8 peptides E T Q P D P G Q I L K K V P M V I G A Y T		(SEQ ID NO: 169)
GGF-I	18	E Y K C L K F K W F K K A T V M		(SEQ ID NO: 17)
GGF-I	19	E A K Y F S K X D A		(SEQ ID NO: 18)
GGF-I	20	E X K F Y V P	LH-alpha	(SEQ ID NO: 19)
GGF-I	21	E L S F A S V R L P G C P P G V D P M V S F P V A L	LH-beta	(SEQ ID NO: 20)

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## FIG. 10

A	GGF-I 01	F K G D A H T E	(SEQ ID NO: 1)
	GGF-I 02	A S L A D E Y E Y M X K	(SEQ ID NO: 22)
	GGF-I 03	T E T S S S G L X L K	(SEQ ID NO: 23)
	GGF-I 07	A S L A D E Y E Y M R K	(SEQ ID NO: 24)
	GGF-I 11	A G Y F A E X A R	(SEQ ID NO: 25)
	GGF-I 13	T T E M A S E Q G A	(SEQ ID NO: 26)
	GGF-I 14	A K E A L A A L K	(SEQ ID NO: 27)
	GGF-I 15	F V L Q A K K	(SEQ ID NO: 28)
	GGF-I 17	E T Q P D P G Q I L K K V P M V I G A Y T	(SEQ ID NO: 29)
	GGF-I 18	E Y K C L K F K W F K K A T V M	(SEQ ID NO: 17)
B	GGF-I 20	E X K F Y V P	(SEQ ID NO: 19)
	GGF-I 12	K L E F L X A K	(SEQ ID NO: 32)

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## FIG. 11

Trypsin peptides			
GGF-II 01	K/R V H Q V W A A K *	(SEQ ID NO: 33)	
GGF-II 02	K/R Y I F F M E P E A X S S G	(SEQ ID NO: 34)	
GGF-II 03	K/R L G A W G P P A F P V X Y	(SEQ ID NO: 35)	
GGF-II 04	K/R W F V V I E G K *	(SEQ ID NO: 36)	
GGF-II 05	K/R A L A A A G Y D V E K *	(SEQ ID NO: 164)	Histone H1
GGF-II 06	K/R L V L R *	(SEQ ID NO: 165)	
GGF-II 07	K/R X X Y P G Q I T S N	(SEQ ID NO: 166)	Trypsin
GGF-II 08	K/R A S P V S V G S V Q E L V Q R *	(SEQ ID NO: 37)	
GGF-II 09	K/R V C L L T V A A P P T	(SEQ ID NO: 38)	
GGF-II 10	K/R D L L L X V	(SEQ ID NO: 39)	
Lysyl Endopeptidase-C peptides			
GGF-II 11	K V H Q V W A A K *	(SEQ ID NO: 51)	
GGF-II 12	K A S L A D S G E Y M X K*	(SEQ ID NO: 52)	

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# FIG. 12

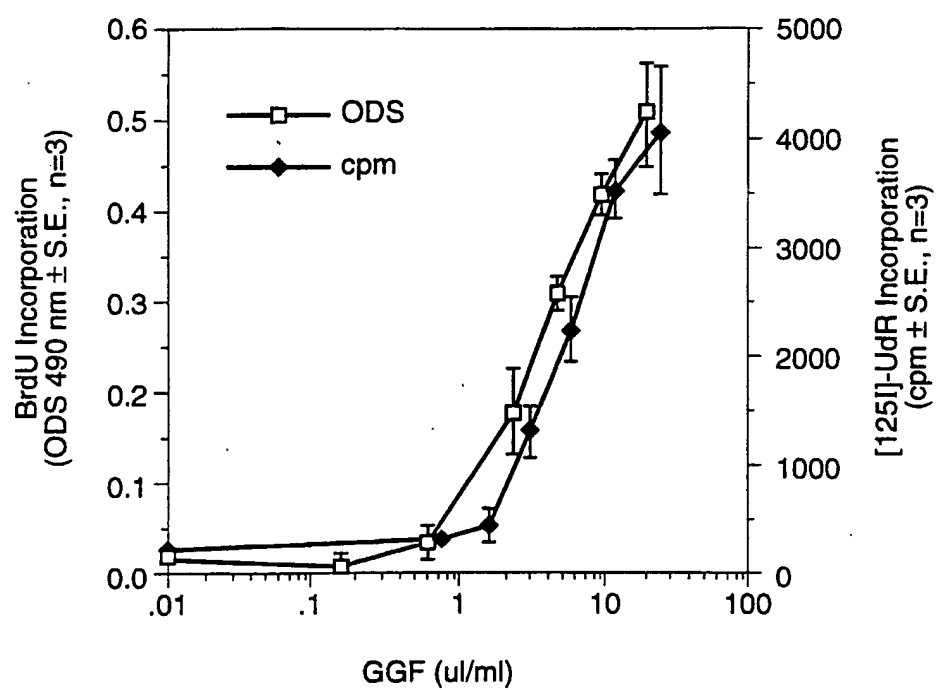
A

GGF-II 01	V H Q V W A A K	(SEQ ID NO: 45)
GGF-II 02	Y I F F M E P E A X S S G	(SEQ ID NO: 46)
GGF-II 03	L G A W G P P A F P V X Y	(SEQ ID NO: 47)
GGF-II 04	W F V V I E G K	(SEQ ID NO: 48)
GGF-II 08	A S P V S V G S V Q E L V Q R	(SEQ ID NO: 49)
GGF-II 09	V C L L T V A A P P T	(SEQ ID NO: 50)
GGF-II 11	K V H Q V W A A K	(SEQ ID NO: 51)
GGF-II 12	K A S L A D S G E Y M X K	(SEQ ID NO: 52)

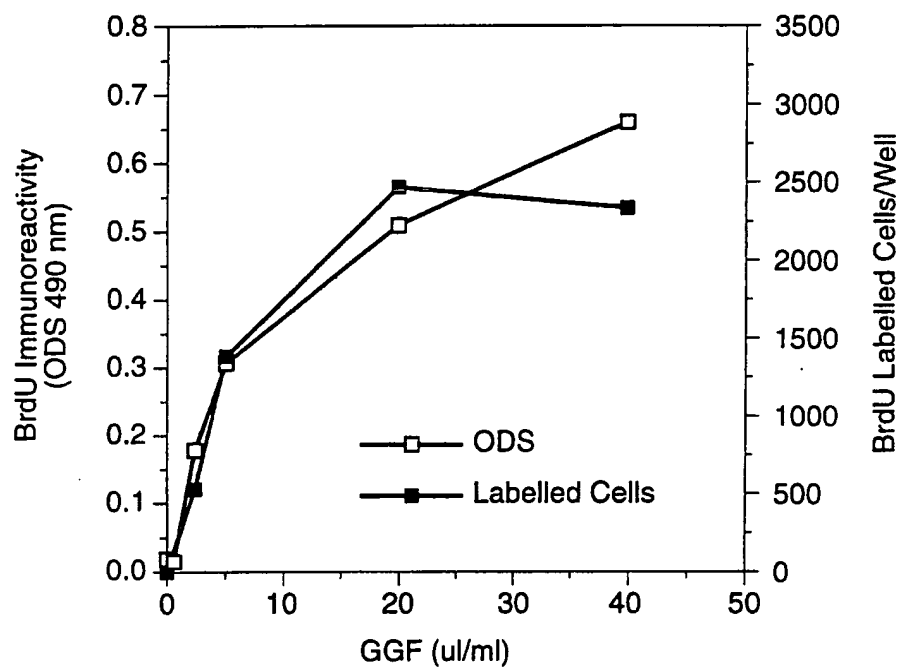
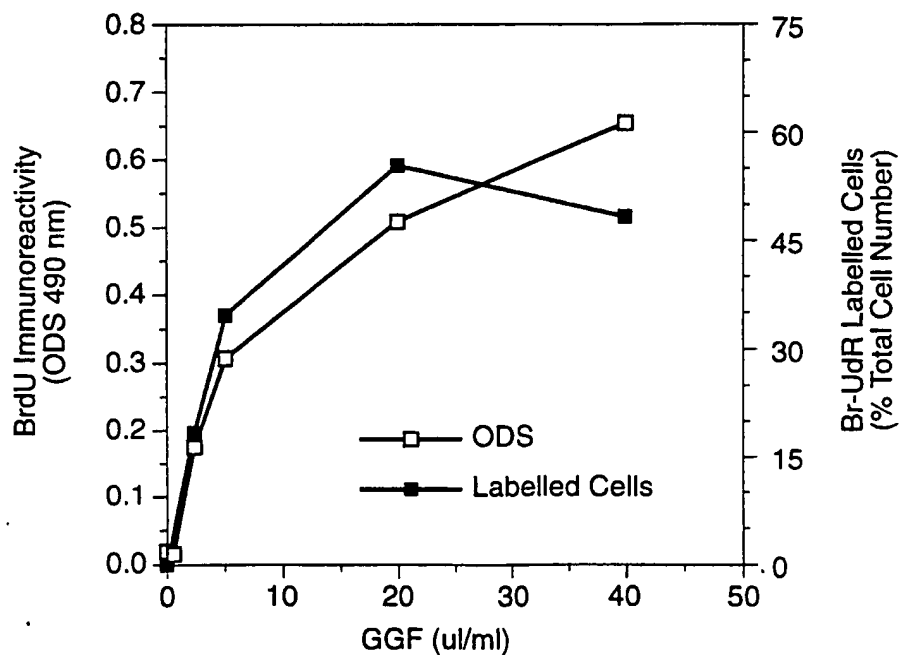
B Novel Factor II Peptides - others

GGF-II 10	D L L L X V	(SEQ ID NO: 53)
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**FIG. 13**

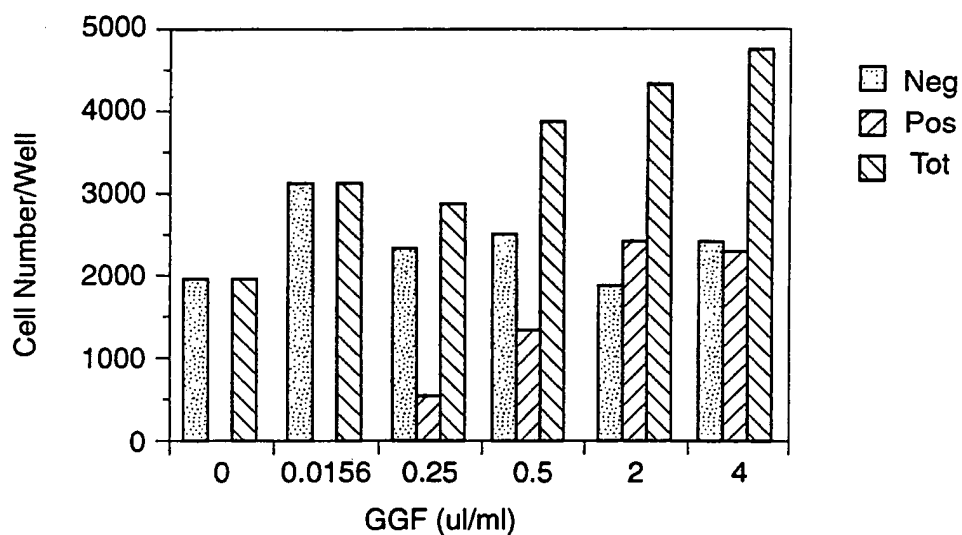
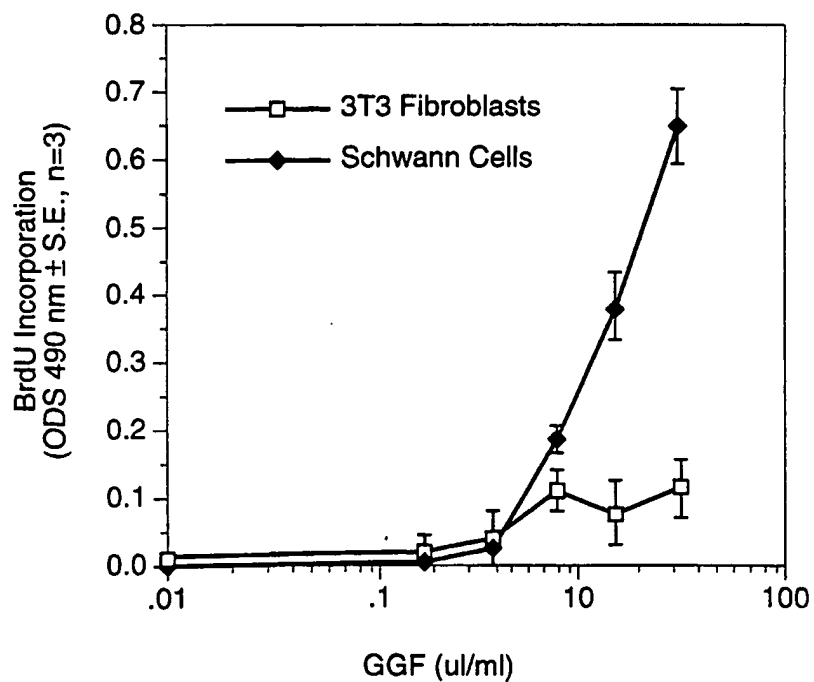
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**FIG. 14A****FIG. 14B**

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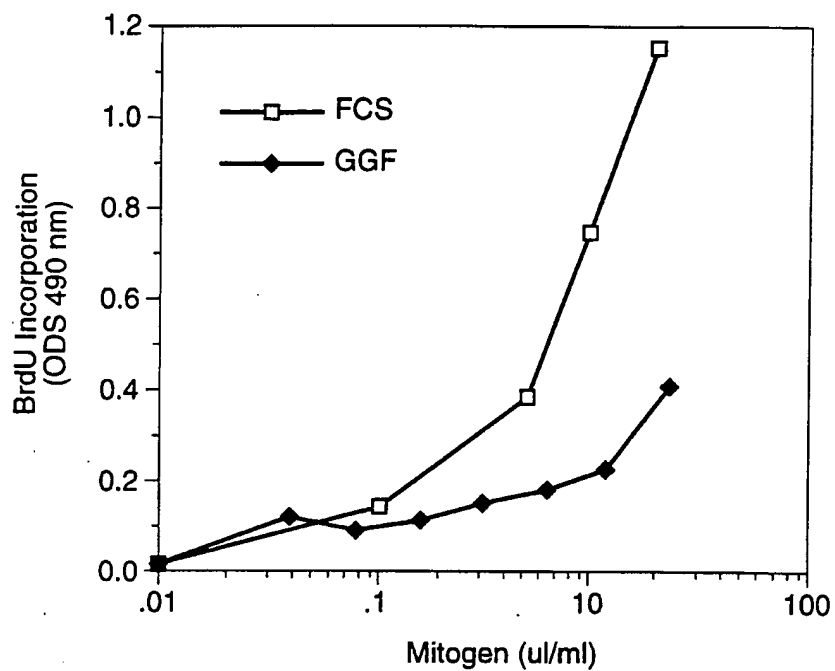
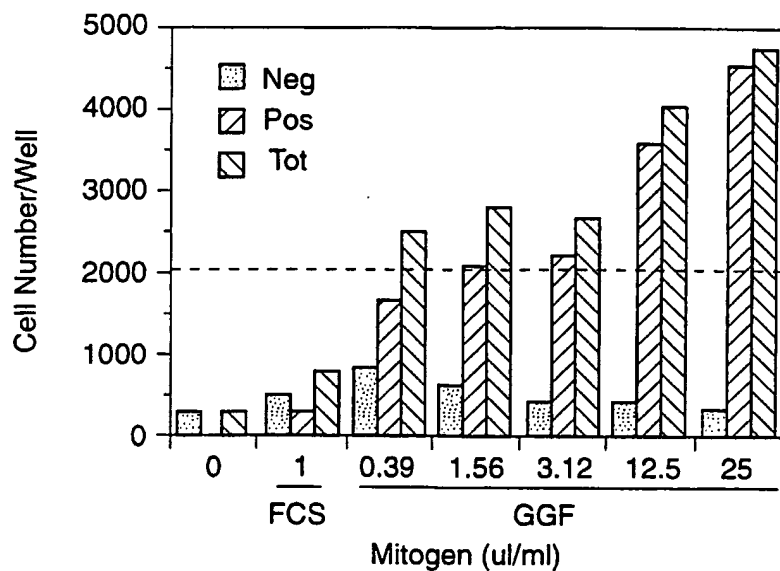


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**FIG. 15****FIG. 16**

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**FIG. 17****FIG. 18**

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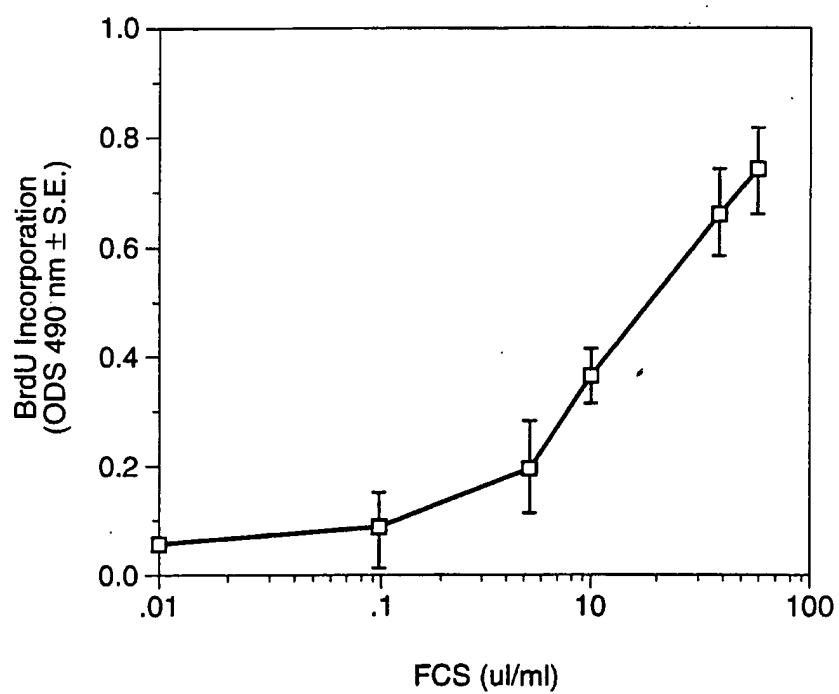
**FIG. 19**

FIG. 20B

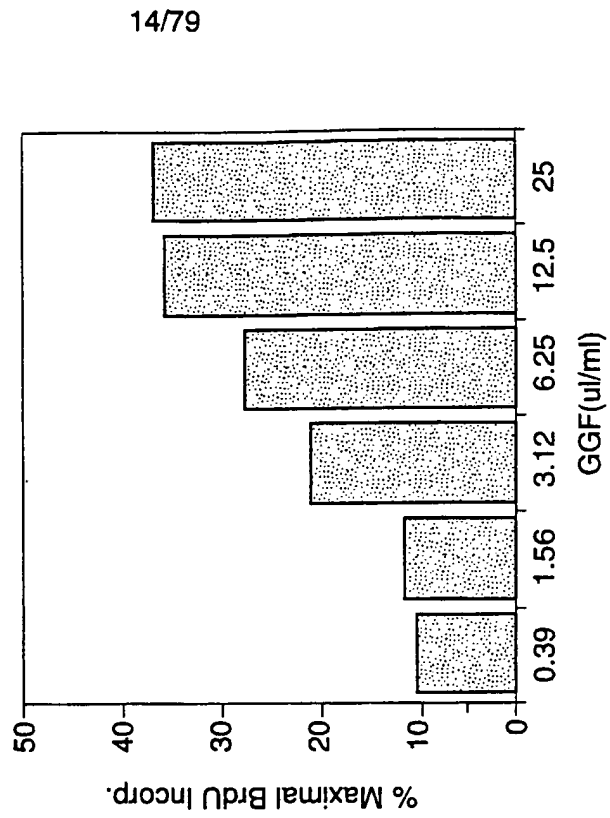
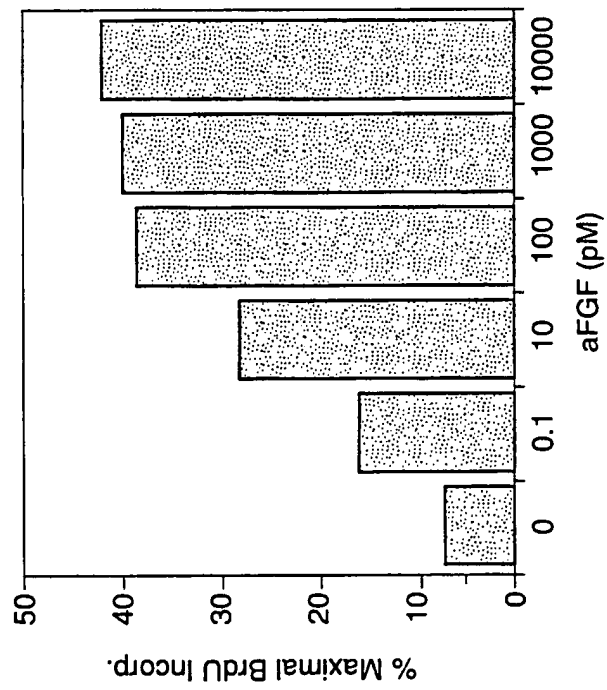


FIG. 20A



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## FIG. 21

Oligo	Sequence	Peptide	
535	TTYAARGGNGAYGCNCAYAC!	GGFI-1	(SEQ ID NO: 54)
536	CATRTAYTCRTAYTCRTCNGC!	GGFI-2	(SEQ ID NO: 55)
537	TGYTCNANGCCATYTCNGT!	GGFI-13	(SEQ ID NO: 56)
538	TGYTCRCTNGCCATYTCNGT!	GGFI-13	(SEQ ID NO: 57)
539	CCDATNACCATNGGNACYTT!	GGFI-17	(SEQ ID NO: 58)
540	GCNGCCCANACYTGRTGNAC!	GGFII-1	(SEQ ID NO: 59)
541	GCYTCNGGYTCCATRAARAA!	GGFII-2	(SEQ ID NO: 60)
542	CCYTCDATNACNACRAACCA!	GGFII-4	(SEQ ID NO: 61)
543	TCNGCRAARTANCCNGC!	GGFI-11	(SEQ ID NO: 62)
544	GCNGCNAGNGCYTCYTTNGC!	GGFI-14	(SEQ ID NO: 63)
545	GCNGCYAANGCYTCYTTNGC!	GGFI-14	(SEQ ID NO: 64)
546	TTYTTNGCYTGNAGNACRAA!	GGFI-15	(SEQ ID NO: 65)
551	TTYTTNGCYTGYAANACRAA!	GGFI-15	(SEQ ID NO: 66)
568	TGNACNAGYTCYTGNA!	GGFII-8	(SEQ ID NO: 67)
569	TGNACYAAYTCYTGNA!	GGFII-8	(SEQ ID NO: 68)
609	CATRTAYTCNCCNGARTCNGC!	GGFII-12	(SEQ ID NO: 69)
610	CATRTAYTCNCCRCTRRTCNGC!	GGFII-12	(SEQ ID NO: 70)
649	NGARTCNGCYAANGANGCYTT!	GGFII-12	(SEQ ID NO: 71)
650	NGARTCNGCNAGNGANGCYTT!	GGFII-12	(SEQ ID NO: 72)
651	RCTRCTCNGCYAANGANGCYTT!	GGFII-12	(SEQ ID NO: 73)
652	RCTRCTCNGCNAGNGANGCYTT!	GGFII-12	(SEQ ID NO: 74)
653	NGARTCNGCYAARCTNGCYTT!	GGFII-12	(SEQ ID NO: 75)
654	NGARTCNGCNAGRCTNGCYTT!	GGFII-12	(SEQ ID NO: 76)
655	RCTRCTCNGCYAARCTNGCYTT!	GGFII-12	(SEQ ID NO: 78)
656	RCTRCTCNGCNAGRCTNGCYTT!	GGFII-12	(SEQ ID NO: 79)
659	ACNACNGARATGGCTCNGA!	GGFI-13	(SEQ ID NO: 80)
660	ACNACNGARATGGCAGYNGA!	GGFI-13	(SEQ ID NO: 81)
661	CAYCARGTNTGGGCNGCNAA!	GGFII-1	(SEQ ID NO: 82)
662	TTYGTNGTNATHGARGGNAA!	GGFII-4	(SEQ ID NO: 83)
663	AARGGNGAYGCNCAYACNGA!	GGFI-1	(SEQ ID NO: 84)
664	GARGCNYTNGCNGCNYTNAA!	GGDI-14	(SEQ ID NO: 85)
665	GTNGGNCTCNGTNCARGARYT!	GGFII-8	(SEQ ID NO: 86)
666	GTNGGNAGYGTNCARGARYT!	GGFII-8	(SEQ ID NO: 87)
694	NACYTTYTTNARHATYTGNC!	GGFI-17	(SEQ ID NO: 88)

## FIG. 22

SEQ ID NO: 89:

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TCCTAA AAC TAC AGA GAC TGT ATT TTC ATG ATC ATA GTT CTG TGA AAT ATA Asn Tyr Arg Asp Cys Ile Phe Met Ile Ile Val Leu Xaa Asn Ile	53
CTT AAA CCG CTT TGG TCC TGA TCT TGT AGG AAG TCA GAA CTT CGC ATT Leu Lys Pro Leu Trp Ser Xaa Ser Cys Arg Lys Ser Glu Leu Arg Ile	101
AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA TAT ATG TGC AAA GTG ATC Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu Ser Met Cys Lys Val Ile	149
AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC AAC ATC ACC ATT GTG GAG Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn Ile Arg Ile Val Glu	197
TCA AAC GGT AAG AGA TGC CTA CTG CGT GCT ATT TCT CAG TCT CTA AGA Ser Asn Gly Lys Arg Cys Leu Leu Arg Ala Ile Ser Gln Ser Leu Arg	245
GGA GTG ATC AAG GTA TGT GGT CAC ACT TGA ATC ACG CAG GTG TGT GAA Gly Val Ile Lys Val Cys Gly His Thr Xaa Ile Thr Gln Val Cys Glu	293
ATC TCA TTG TGA ACA AAT AAA AAT CAT GAA AGG AAA ACT CTA TGT TTG Ile Ser Cys Xaa Thr Asn Lys Asn His Glu Arg Lys Thr Leu Cys Leu	341
AAA TAT CTT ATG GGT CCT CCT GTA AAG CTC TTC ACT CCA TAA GGT GAA Lys Tyr Leu Met Gly Pro Pro Val Lys Leu Phe Thr Pro Xaa Gly Glu	389
ATA GAC CTG AAA TAT ATA TAG ATT ATT T Ile Asp Leu Lys Tyr Ile Xaa Ile Ile	417

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## FIG. 23A

## Degenerate PCR Primers

Oligo Sequence	Peptide	(SEQ ID NO: 90)
657 CCGAATTCTGCAGGARACNCARCCNGAYCCNNGG!	GGFI-17	(SEQ ID NO: 90)
658 AAGGATCCTGCAGNGTRTANGCNCCHATNACCATNNGG!	GGFI-17	(SEQ ID NO: 91)
667 CCGAATTCTGCAGGCNGAYTCNNGNGARTAYATG!	GGFII-12	(SEQ ID NO: 92)
668 CCGAATTCTGCAGGCNGAYATYGGNGARTAYAT!	GGFII-12	(SEQ ID NO: 93)
669 AAGGATCCTGCAGNCCNCAATRTAYTCNCCNGARTC!	GGFII-12	(SEQ ID NO: 94)
670 AAGGATCCTGCAGNCCNCAATRTAYTCNCCRRTRTC!	GGFII-12	(SEQ ID NO: 95)
671 CCGAATTCTGCAGCAYCARGTNTGGGCGCNAA!	GGFII-1	(SEQ ID NO: 96)
672 CCGAATTCTGCAGATRTTYTYATGGARCCNGARG!	GGFII-2	(SEQ ID NO: 97)
673 CCGAATTCTGCAGGGGNCNCCNGCNTTYCCNGT!	GGFII-3	(SEQ ID NO: 98)
674 CCGAATTCTGCAGTGGTTYGTNGTNATHGARGG!	GGFII-4	(SEQ ID NO: 99)
677 AAGGATCCTGCAGYTTNGCNGCCCANACYTGRTG!	GGFII-1	(SEQ ID NO: 100)
678 AAGGATCCTGCAGGCTCNGGYTCCATRAARAA!	GGFII-2	(SEQ ID NO: 101)
679 AAGGATCCTGCAGACNGGRAANGCNGGNGNCC!	GGFII-3	(SEQ ID NO: 102)
680 AAGGATCCTGCAGYTTNCCYTCDATNACNACRAAC!	GGFII-4	(SEQ ID NO: 103)
681 CATRTAYTCRTAYTCTCNGCAAGGATCCTGCAG!	GGFI-2	(SEQ ID NO: 104)
682 CCGAATTCTGCAGAARGNGAYGCNCAYACNGA!	GGFI-1	(SEQ ID NO: 105)
683 GCNGCYAANGCYRCYTTNGCAAGGATCCTGCAG!	GGFI-14	(SEQ ID NO: 106)
684 GCNGCNAGNGCYTCYTTNGCAAGGATCCTGCAG!	GGFI-14	(SEQ ID NO: 107)
685 TCNGCRAARTANCCNGCAAGGATCCTGCAG!	GGFII-1	(SEQ ID NO: 108)

# FIG. 23B

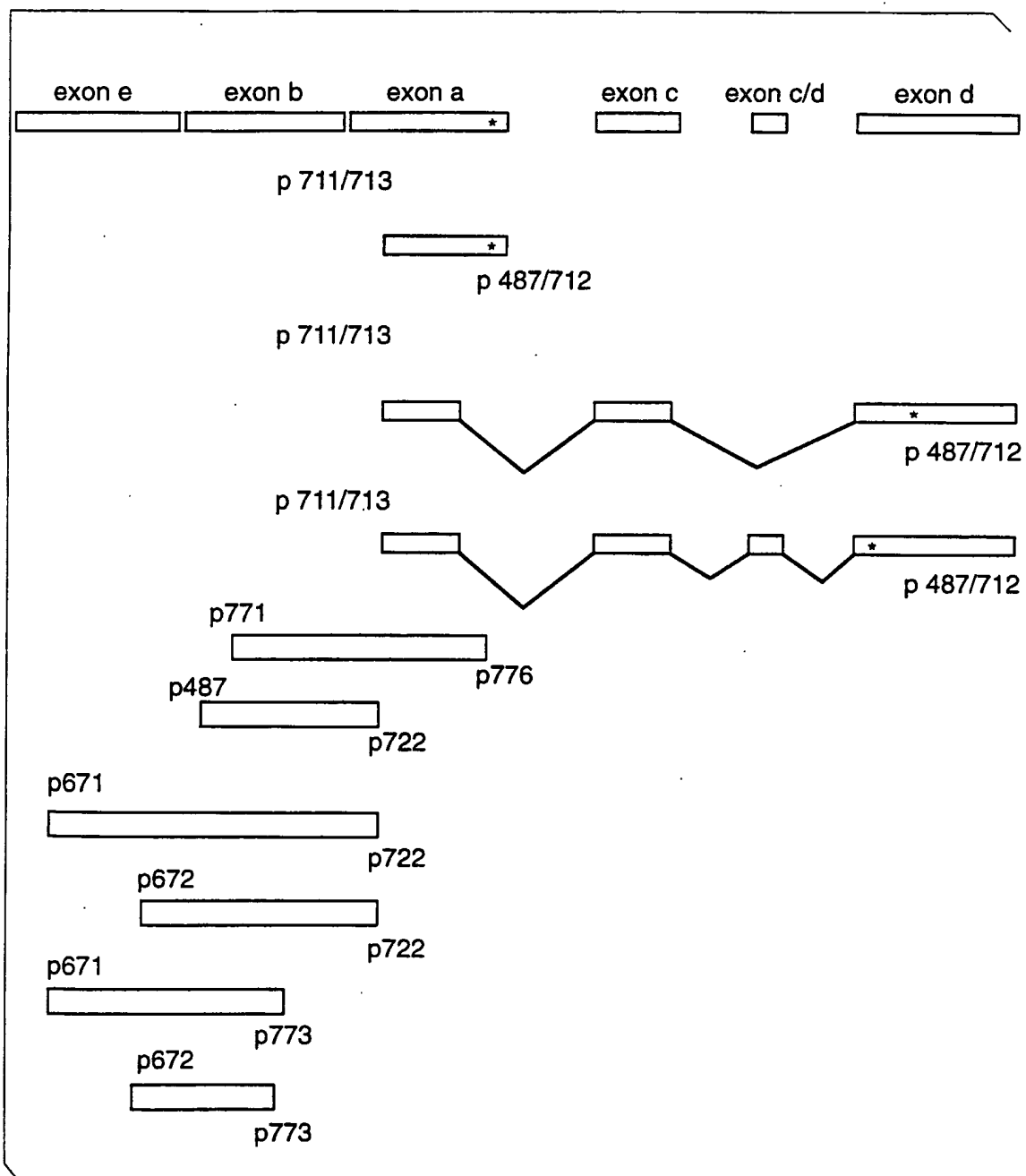
## Unique PCR Primers for Factor II

Oligo Sequence	Comment	(SEQ ID NO: 109)
711 CATCGATCTGCAGGCTGATTCTCTGGAGAATATATGTGCA!	3' RACE	(SEQ ID NO: 110)
712 AAGGATCCTGCAGCCACATCTCGAGTCGACATCGATT!	3' RACE	(SEQ ID NO: 111)
713 CCGAATTCTGCAGTGATCAGCAAACTAGGAAATGACA!	3' RACE	(SEQ ID NO: 112)
721 CATCGATCTGCAGCCTAGTTTGTGCTGATCACTTTGCAC!	5' RACE	(SEQ ID NO: 113)
722 AAGGATCCTGCAGTATATTCTCCAGAATCAGCCAGTG!	5' RACE; ANCHORED	(SEQ ID NO: 114)
725 AAGGATCCTGCAGGCACCGCAGTAGGCATCTCTTA!	EXON A	(SEQ ID NO: 115)
726 CCGAATTCTGCAGCAGAACTTCGCATTAGCAAAGC!	EXON A	(SEQ ID NO: 116)
771 CATCCCGGGATGAAGAGTCAGGAGTCTGTGGCA!	EXONS B+A	(SEQ ID NO: 117)
772 ATACCCGGGCTGCAGACAAATGAGATTTACACACACCTGCG!		(SEQ ID NO: 118)
773 AAGGATCCTGCAGTTTGGAACTGCCACAGACTCCT!	ANCHORED	(SEQ ID NO: 119)
776 ATACCCGGGCTGCAGATGAGATTTACACACACCTGCGTGA!	EXONS B+A	

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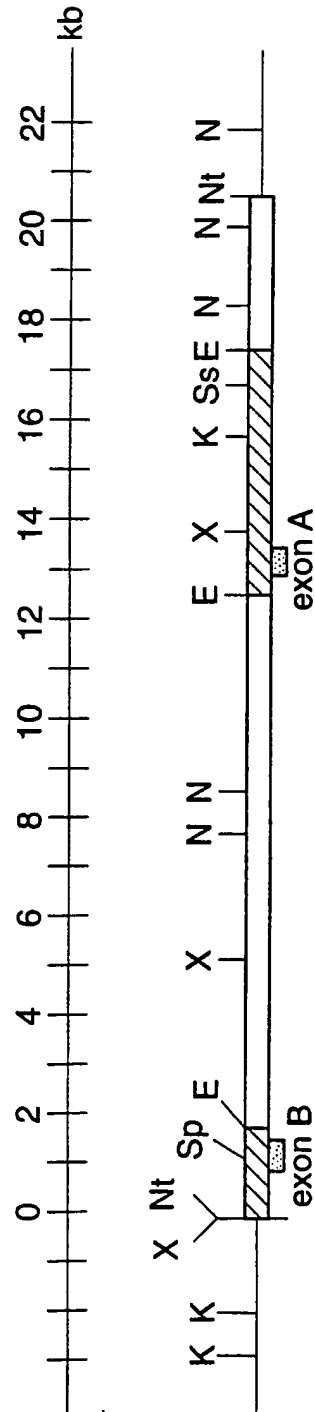


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**FIG. 24**

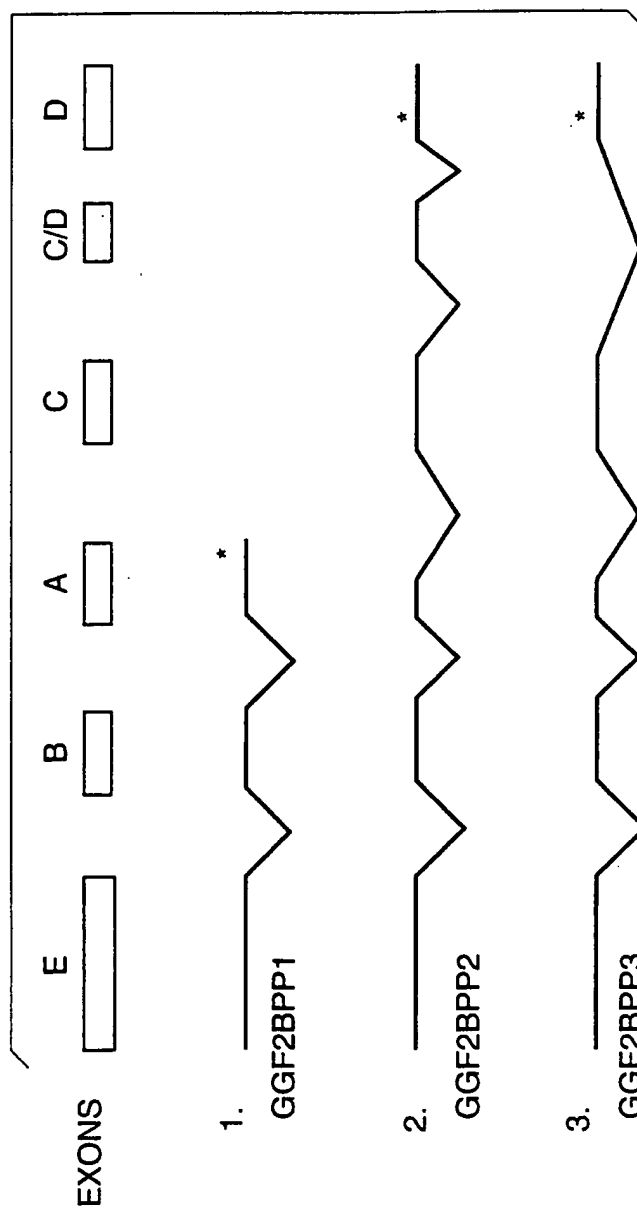
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FIG. 25



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FIG. 26



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**FIG. 27**

Peptide	Pos.	Sequence match	ID Sequences
II-1	1:	VHQVWAAK HQVWAAK AAGLK	(SEQ ID NO:120)
II-10	14:	DLLLV GGLKK dslltv RLGAW	(SEQ ID NO:121)
II-03	21:	LGAWGPPAFPVXY LLTVR lgawghpafpscgl RLKED	(SEQ ID NO:122) (SEQ ID NO:123)
II-02	41:	YIFFMEPEAXSSG KEDSR YIFFMEPEANSSG GPGRL	(SEQ ID NO:124) (SEQ ID NO:125)
II-6	103:	LVLR VAGSK LVLR CETSS	(SEQ ID NO:126)
I-18	112:	EYKCLKFKWFKKATVM CETSS eysslkfkfwfkngsel SRKNK	(SEQ ID NO:127) (SEQ ID NO:128)
II-12	151:	KASLADSGEYMXK ELRIS KASLADSGEYMCK VISKL	(SEQ ID NO:129) (SEQ ID NO:130)
I-07	152:	ASLADEYEYMRK LRISK asladsgeymck VISKL	(SEQ ID NO:131) (SEQ ID NO:132)

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**FIG. 28A**

SEQ ID NO: 133:

CCTGCAG CAT CAA GTG TGG GCG AAA GCC GGG GGC TTG AAG AAG GAC TCG CTG 55  
     His Gln Val Trp Ala Ala Lys Ala Gly Gly Leu Lys Lys Asp Ser Leu  
 CTC ACC GTG CGC CTG GGC GCC TGG GGC CAC CCC GGC TTC CCC TCC TGC 103  
     Leu Thr Val Arg Leu Gly Ala Trp Gly His Pro Ala Phe Pro Ser Cys  
 GGG CGC CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC GAG 151  
     Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro Glu  
 GCC AAC AGC AGC GGC GGG CCC GGC CGC CTT CCG AGC CTC CTT CCC CCC 199  
     Ala Asn Ser Ser Gly Gly Pro Gly Arg Leu Pro Ser Leu Leu Pro Pro  
 TCT CGA GAC GGG CCG GAA CCT CAA GAA GGA GGT CAG CCG GGT GCT GTG 247  
     Ser Arg Asp Gly Pro Glu Pro Gln Glu Gly Gly Gln Pro Gly Ala Val  
 CAA CGG TGC GCC TTG CCT CCC CGC TTTG AAA GAG ATG AAG AGT CAG GAG 295  
     Gln Arg Cys Ala Leu Pro Pro Arg Leu Lys Glu Met Lys Ser Gln Glu  
 TCT GTG GCA GGT TCC AAA CTA GTG CTT CGG TGC GAG ACC AGT TCT GAA 343  
     Ser Val Ala Gly Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu  
 TAC TCC TCT CTC AAG TTC AAG TGG Trp Phe Lys Asn Gly Ser Glu Leu Ser 391  
     Tyr Ser Ser Leu Lys Lys Phe Lys Trp Phe Lys Asn Gly Ser Glu Leu Ser  
 CGA AAG AAC AAA CCA GAA AAC ATC AAG ATA CAG AAA AGG CCG GGG AAG 439  
     Arg Lys Asn Lys Pro Glu Asn Ile Lys Ile Gln Lys Arg Pro Gly Lys  
 TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA TAT 487  
     Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu Tyr  
 ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC AAC 535  
     Met Cys Lys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn  
 ATC ACC ATT GTG GAG TCA AAC GGT AAG AGA TGC CTA CTG CGT GCT ATT 583  
     Ile Thr Ile Val Glu Ser Asn Gly Lys Arg Cys Leu Leu Arg Ala Ile  
 TCT CAG TCT CTA AGA GGA GTG ATC AAG GTA TGT GGT CAC ACT 625  
     Ser Gln Ser Leu Arg Gly Val Ile Lys Val Cys Gly His Thr  
 TGAATCACGC AGGTGTGTGA AATCTCATTTG TGAACAAATA AAAATCATGA AAGGAAAAA 685  
     AAAAAAAAA AATCGATGTC GACTCGAGAT GTGGCTGCAG GTCGACTCTA GAGGATCCC 744

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## FIG. 28B

SEQ ID NO: 134:

CCTGCAG CAT CAA GTG TGG GCG GCG AAA GCC GGG GGC TTG AAG AAG GAC TCG CTG	55
His Gln Val Trp Ala Ala Lys Ala Gly Gly Leu Lys Lys Asp Ser Leu	
CTC ACC GTG CGC CTG GGC GGC TGG GGC CAC CCC GGC TTC CCC TCC TGC	103
Leu Thr Val Arg Leu Leu Gly Ala Trp Gly His Pro Ala Phe Pro Ser Cys	
GGG CGC CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC GAG	151
Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro Glu	
GCC AAC AGC AGC GGC GGC GGC CCC GGC CTT CCG AGC CTC CTT CCC CCC	199
Ala Lys Ser Ser Gly Gly Pro Gly Arg Leu Pro Ser Leu Leu Pro Pro	
TCT CGA GAC GGC CCG GAA CCT CAA GAA GGA GGT CAG CCG GGT GCT GTG	247
Ser Arg Asp Gly Pro Glu Pro Gln Glu Gly Gly Gln Pro Gly Ala Val	
CAA CGG TGC GCC TTT GGC CCT CCC CGC TTT AAA GAG ATG AAG AGT CAG GAG	295
Gln Arg Cys Ala Leu Leu Pro Pro Arg Leu Lys Glu Met Lys Ser Gln Glu	
TCT GTG GCA GGT TCC AAA CTA GTG CTT CCG TGC GAG ACC AGT TCT GAA	343
Ser Val Ala Ala Gly Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu	
TAC TCC TCT CTC AAG TTT AAG TGG TTT AAG AAT GGG AGT GAA TTA AGC	391
Tyr Ser Ser Leu Lys Lys Phe Lys Trp Phe Lys Asn Gly Ser Glu Leu Ser	
CGA AAG AAC AAA CCA GAA AAC ATC AAG ATA CAG AAA AGG CCG GGG AAG	439
Arg Lys Asn Lys Gly Gly Asn Ile Lys Ile Gln Lys Arg Pro Gly Lys	
TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA TAT	487
Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu Tyr	
ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC AAC	535
Met Cys Lys Val Ile Ser Lys Leu Gly Lys Asn Asp Ser Ala Ser Ala Asn	

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## FIG. 28B'

ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG ACA  
 Ile Thr Ile Val Glu Ser Asn Ala Thr Ser Thr Ser Thr Ala Gly Thr 583  
 AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT  
 Ser His Leu Val Lys Ser Ala Glu Lys Glu Lys Thr Phe Cys Val Asn 631  
 GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC  
 Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr 679  
 TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT  
 Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn 727  
 GTG CCC ATG AAA GTC CAA ACC CAA GAA AGT GCC CAA ATG AGT TTA CTG  
 Val Pro Met Lys Val Gln Thr Gln Glu Ser Ala Gln Met Ser Leu Leu 775  
 GTG ATC GCT GCC AAA ACT ACG TAATGGCCAG CTTCTACAGT ACGTCCACTC  
 Val Ile Ala Ala Lys Thr Thr 826  
 CCTTTCTGTC TCTGCCTGAA TAGCGCATCT CAGTCGGTGC CGCTTTCTTG TTGCCGCATC 886  
 TCCCCTCAGA TTCCTCCTAG AGCTAGATGC GTTTTACCAG GTCTAACATT GACTGCCCTCT 946  
 GCCTGTGCGA TGAGAACATT AACACAAGCG ATTGTATGAC TTCCCTCTGTC CGTGACTAGT 1006  
 GGGCTCTGAG CTACTCGTAG GTGCGTAAGG CTCCAGTGTT TCTGAAATTG ATCTTGAATT 1066  
 ACTGTGATAC GACATGATAG TCCCTCTCAC CCAGTGCAAT GACAATAAAG GCCTTGAAAA 1126  
 GTCAAAAAAA AAAAAAAAAA AAAAAATCGA TGTCGACTCG AGATGTGGCT GCAGGTCGAC 1186  
 TCTAGAG 1193

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## FIG. 28C

SEQ ID NO: 135:

CCTGCAG	CAT	CAA	GTG	TGG	GCG	GCG	AAA	GCC	GGG	GCG	GGC	TTG	AAG	AAG	GAC	TCG	CTG	55
	His	Gln	Val	Trp	Ala	Ala	Lys	Ala	Gly	Gly	Gly	Leu	Lys	Lys	Asp	Ser	Leu	
CTC	ACC	GTG	CGC	CTG	GGC	GCC	TGG	GGC	CAC	CCC	GCC	TTC	CCC	TCC	TGC			103
Leu	Thr	Val	Arg	Leu	Gly	Ala	Trp	Gly	His	Pro	Ala	Phe	Pro	Ser	Cys			
GGG	CGC	CTC	AAG	GAG	GAC	AGC	AGG	TAC	ATC	TTC	TTC	ATG	GAG	CCC	GAG			151
Gly	Arg	Leu	Lys	Glu	Asp	Ser	Arg	Tyr	Ile	Phe	Phe	Met	Glu	Pro	Glu			
GCC	AAC	AGC	AGC	GGC	GGG	CCC	GGC	CGC	CTT	CCG	AGC	CTC	CTT	CCC	CCC			199
Ala	Asn	Ser	Ser	Gly	Gly	Pro	Gly	Arg	Leu	Pro	Ser	Leu	Leu	Pro	Pro			
TCT	CGA	GAC	GGG	CCG	GAA	CCT	CAA	GAA	GGA	GGT	CAG	CCG	GGT	GCT	GTG			247
Ser	Arg	Asp	Gly	Pro	Glu	Pro	Gln	Glu	Gly	Gly	Gln	Pro	Gly	Ala	Val			
CAA	CGG	TGC	GCC	TTG	CCT	CCC	CGC	TTG	AAA	GAG	ATG	AAG	AGT	CAG	GAG			295
Gln	Arg	Cys	Ala	Leu	Pro	Pro	Arg	Leu	Lys	Glu	Met	Lys	Ser	Gln	Glu			
TCT	GTG	GCA	GGT	TCC	AAA	CTA	GTG	CTT	CGG	TGC	GAG	ACC	AGT	TCT	GAA			343
Ser	Val	Ala	Gly	Ser	Lys	Leu	Val	Leu	Arg	Cys	Glu	Thr	Ser	Ser	Glu			
TAC	TCC	TCT	CTC	AAG	TTC	AAG	TGG	TTC	AAG	AAT	GGG	AGT	GAA	TTA	AGC			391
Tyr	Ser	Ser	Leu	Lys	Phe	Lys	Trp	Phe	Lys	Asn	Gly	Ser	Glu	Leu	Ser			
CGA	AAG	AAC	AAA	CCA	GAA	AAC	ATC	AAG	ATA	CAG	AAA	AGG	CCG	GGG	AAG			439
Arg	Lys	Asn	Lys	Pro	Glu	Asn	Ile	Lys	Ile	Gln	Lys	Arg	Pro	Pro	Lys			
TCA	GAA	CTT	CGC	ATT	AGC	AAA	GCG	TCA	CTG	GCT	GAT	TCT	GGA	GAA	TAT			487
Ser	Glu	Leu	Arg	Ile	Ser	Lys	Ala	Ser	Leu	Ala	Asp	Ser	Gly	Glu	Tyr			



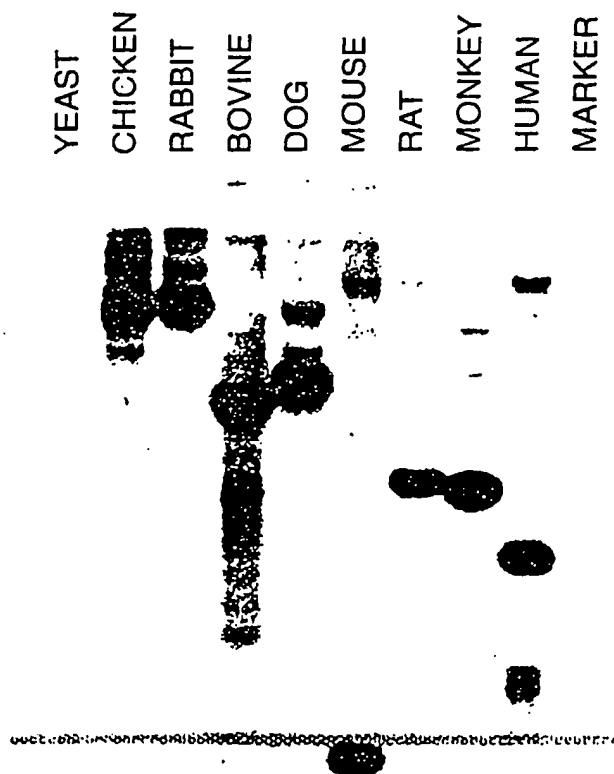
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## FIG. 28C'

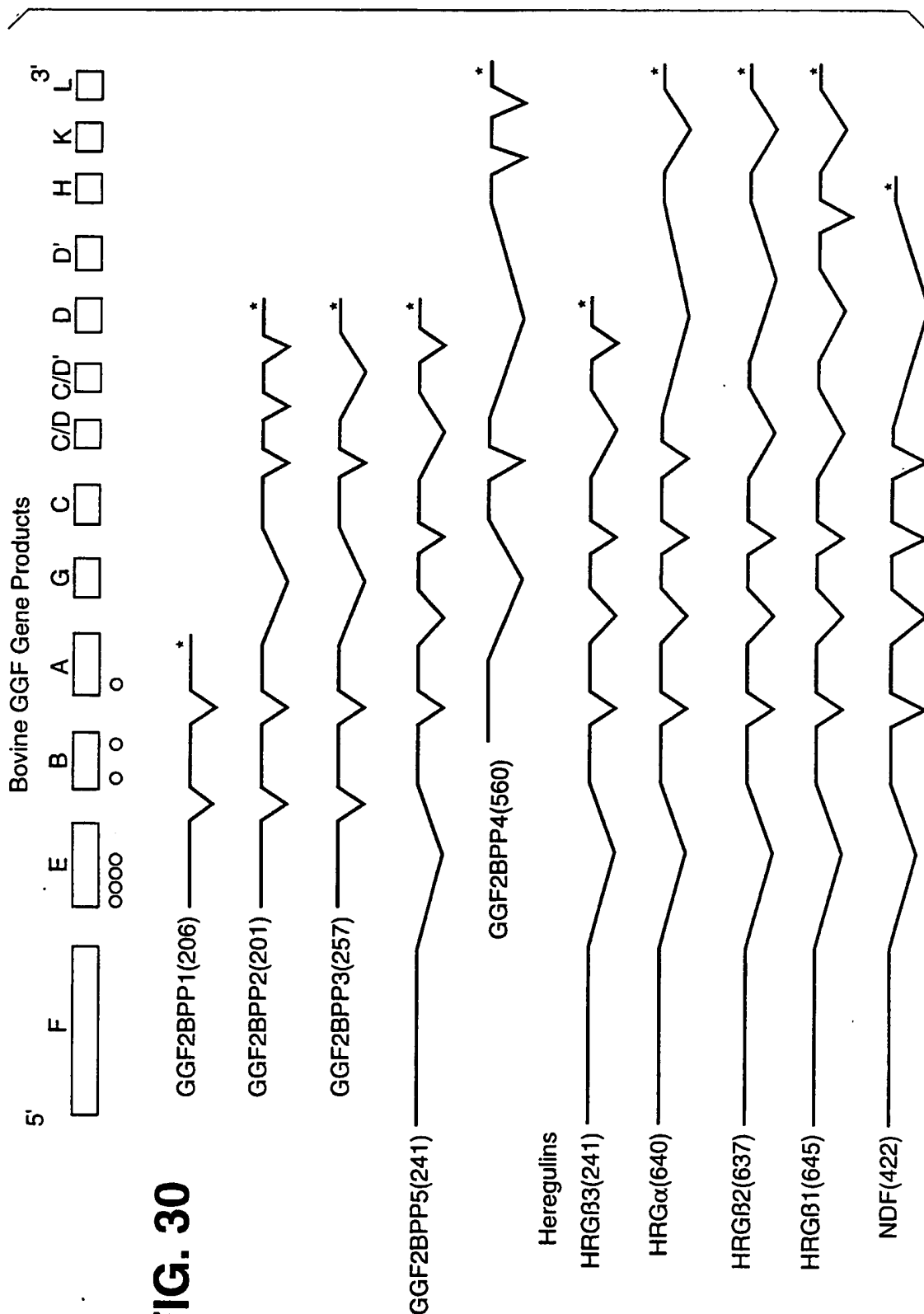
535 ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC AAC  
 Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn  
 583 ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG ACA  
Ile Arg Ile Val Glu Ser Asn Ala Thr Ser Thr Ser Thr Ala Gly Thr  
 631 AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT  
 Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn  
 679 GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC  
 Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr  
 727 TTG TGC AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC  
 Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr  
 775 GTA ATG GCC AGC TTC TAC AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT  
 Val Met Ala Ser Phe Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro  
 838 GAA TAGCGCATCT CAGTCGGTGC CGCTTTCTTG TTGCCGCATC TCCCCCTCAGA TTCCGGCCTAG  
 Glu  
 898 AGCTAGATGC GTTTTACCAG GTCTAACATT GACTGCCCTCT GCCTGTGCGCA TGAGAACATT  
 958 AACACAAGCG ATTGTAATGAC TTCCCTCTGTC CGTGACTAGT GGGCTCTGAG CTACTCGTAG  
 1018 GTCCGTAAGG CTCCAGTGTT TCTGAAATTG ATCTTGAATT ACTGTGATAC GACATGATAG  
 1078 TCCCTCTCAC CCAGTGCAAT GACAATAAAG GCCTTGAAAA GTCAAAAAAAA AAAAAAAA  
 1108 AAAAAATCGAT GTCGACTCGA GATGTGGCTG

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**FIG. 29**



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**FIG. 30**

**FIG. 31A**

CODING SEGMENT F: (SEQ ID NO: 136 (bovine) and 173 (human))

AGT<sup>TT</sup>CCCCC CCCAACT<sup>T</sup>TGT CGGA<sup>ACT</sup>CTG GGT<sup>CG</sup>CGCG CAGGCAGGA GCGAGCGGC 60

CGCGGGCTGCC CAGCGATGC GAGCGGGC CGACGGTAA TCGCCTCTCC CTCCTCGGGC 120

TGCGAGCGCG CCGACCGAG GCAGCGACAG GAGCGGACCG CGGCGGAAC CGAGGACTCC 180

CCAGCGGGCG GCCAGCAGGA GCCACCCCGC GAGNCGTGCG ACCGGGACGG AGCGCCCGCC 240

AGTCCCAGGT GGCCCGGACC GCACGTTGCG TCCCCGCGCT CCCC GCCCGC GACAGGAGAC 300

GGTCCCCCCC ACGCCGGCGG CGCCTCGGCC CGGTGGCTGG CCGGCCTCCA CTCCGGGGAC 360

[illegible]

AAACCTTTCC CGAAGCCGAT CCAAGCCCTC GGACCCAAAC TTGTCGCGG TCGCCTTCGC 420

AAACTTTTCC CAAACCCGAT CCGAGCCCTT GGACCAA.. .....C TCGCCTGCGC

Met Ser Glu Arg Arg

CCGGAGCCGT CCGGCAGAG CGTGCACTTC TCGGCGGAG ATG TCG GAG CGC AGA 474

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GAA GGC AAA GGC AAG GGG AAG GGC AAG AAG GAC CGA GGC TCC GGG

522

**Figure 1**

R K E

Pys Lys Pys Pys Pys Pys Pys Pys Pys Pys  
AAG AAG AAG CCC GTG CCC GCG GGT GGC  
GGC CCG AGC CCA C

550

[illegible]

# SE

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## FIG. 31B

CODING SEGMENT E: (SEQ ID NO: 137)

CC CAT CAN GTG TGG GCG GCG AAA GCC GGC GGC TTG AAG AAG GAC TCG	47
His Gln Val Trp Ala Ala Lys Ala Gly Gly Lys Lys Asp Ser	
CTG CTC ACC GTG CGC CTG GGC GCC TGG GGC CAC CCC TTC CCC TCC	95
Leu Leu Thr Val Arg Leu Gly Ala Trp Gly His Pro Ala Phe Pro Ser	
TGC GGG CGC CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC	143
Cys Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro	
GAG GCC AAC AGC AGC GGC GGC CCC GGC CTT CCG AGC CTC CTT CCC	191
Glu Ala Asn Ser Ser Gly Gly Pro Gly Arg Leu Pro Ser Leu Leu Pro	
CCC TCT CGA GAC GGG CCG GAA CCT CAA GAA GGA GGT CAG CCG GGT GCT	239
Pro Ser Arg Asp Gly Pro Glu Pro Gln Glu Gly Gly Gln Pro Gly Ala	
GTG CAA CGG TGC G	252
Val Gln Arg Cys	

## FIG. 31C

CODING SEGMENT B: (SEQ ID NO: 138 (bovine) and 174 (human))

Leu	Pro	Pro	Arg	Leu	Lys	Glu	His	Lys	Ser	Gln	Glu	Ser	Val	Ala	Gly	
CCT	TGC	CTC	CCC	GCT	TGA	AAG	AGA	TGA	AGA	GTC	AGG	AGT	CTG	TGG	CAG	48
CCT	TGC	CTC	CCC	GAT	TGA	AAG	AGA	TGA	AAA	GCC	AGG	AAT	CGG	CTG	CAG	
Ser	Lys	Leu	Val	Leu	Arg	Cys	Glu	Thr	Ser	Ser	Glu	Tyr	Ser	Ser	Leu	
GTT	CCA	AAC	TAG	TGC	TTC	GGT	GCG	AGA	CCA	GTT	CTG	AAT	ACT	CCT	CTC	96
GTT	CCA	AAC	TAG	TCC	TTC	GGT	GTG	AAA	CCA	GTT	CTG	AAT	ACT	CCT	CTC	
Lys	Phe	Lys	Trp	Phe	Lys	Asn	Gly	Ser	Glu	Leu	Ser	Arg	Lys	Asn	Lys	
TCA	AGT	TCA	AGT	GGT	TCA	AGA	ATG	GGA	GTG	AAT	TAA	GCC	GAA	AGA	ACA	144
TCA	GAT	TCA	AGT	GGT	TCA	AGA	ATG	GGA	ATG	AAT	TGA	ATC	GAA	AAA	ACA	
Pro	Gly	Asn	Ile	Lys	Ile	Gln	Lys	Arg	Pro	Gly						
AAC	CAC	AAA	ACA	TCA	AGA	TAC	AGA	AAA	GGC	CGG	G					178
AAC	CAC	AAA	ATA	TCA	AGA	TAC	AAA	AAA	AGC	CAG	G					

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## FIG. 31D

CODING SEGMENT A: (SEQ ID NO: 139 (bovine) and 175 (human))

Lys	Ser	Glu	Leu	Arg	Ile	Ser	Lys	Ala	Ser	Leu	Ala	Asp	Ser	Gly	46	
G	AAG	TCA	GAA	CTT	CGC	ATT	AGC	AAA	GCG	TCA	CTG	GCT	GAT	TCT	GGA	
G	AAG	TCA	GAA	CTT	CGC	ATT	AAC	AAA	GCA	TCA	CTG	GCT	GAT	TCT	GGA	
N																
Glu	Tyr	Met	Cys	Lys	Val	Ile	Ser	Lys	Leu	Gly	Asn	Asp	Ser	Ala	Ser	
GAA	TAT	ATG	TGC	AAA	GTG	ATC	AGC	AAA	CTA	GGA	AAT	GAC	AGT	GCC	TCT	
GAG	TAT	ATG	TGC	AAA	GTG	ATC	AGC	AAA	TTA	GGA	AAT	GAC	AGT	GCC	TCT	
94																
Ala	Asn	Ile	Thr	Ile	Val	Glu	Ser	Asn	Ala							122
GCC	AAC	ATC	ACC	ATT	GTG	GAG	TCA	AAC	G							
GCC	AAT	ATC	ACC	ATC	GTG	GAA	TCA	AAC	G							

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## FIG. 31E

CODING SEGMENT A': (SEQ ID NO: 140)

TCTAAAACTA CAGAGACTGT ATTTTCATGA TCATCATAGT TCTGTGAAAT ATACTTAAAC	60
CGCTTTGGTC CTGATCTTGT AGG AAG TCA GAA CTT CGC ATT AGC AAA GCG	110
Lys Ser Glu Leu Arg Ile Ser Lys Ala	
TCA CTG GCT GAT TCT GGA GAA TAT ATG TGC AAA GTG ATC AGC AAA CTA	158
Ser Leu Ala Asp Ser Gly Glu Tyr Met Cys Lys Val Ile Ser Lys Leu	
GGA AAT GAC AGT GCC TCT GCC AAC ATC ACC ATT GTG GAG TCA AAC GGT	206
Gly Asn Asp Ser Ala Ser Ala Asn Ile Thr Ile Val Glu Ser Asn Gly	
AAG AGA TGC CTA CTG CGT GCT ATT TCT CAG TCT CTA AGA GGA GTG ATC	254
Lys Arg Cys Leu Leu Arg Ala Ile Ser Gln Ser Leu Arg Gly Val Ile	
AAG GTA TGT GGT CAC ACT TGAATCACGC AGGTGTGTGA AATCTCATTG	302
Lys Val Cys Gly His Thr	
TGAACAAATA AAAATCATGA AAGGAAAACT CTATGTTTGA AATATCTTAT GGGTCCTCCT	362
GTAAGCTCT TCACTCCATA AGGTGAAATA GACCTGAAAT ATATATAGAT TATTT	417



**FIG. 31F**

CODING SEGMENT G: (SEQ ID NO: 141 (bovine) and 176 (human))

Glu	Ile	Thr	Thr	Thr	Gly	Met	Pro	Ala	Ser	Thr	Glu	Thr	Ala	Tyr	Val	Ser
AG	ATC	ACC	ACT	GGC	ATG	CCA	GCC	TCA	ACT	GAG	ACA	GCG	TAT	GTG	TCT	TCT
AG	ATC	ATC	ACT	GGT	ATG	CCA	GCC	TCA	ACT	GAA	GGA	GCA	TAT	GTG	GTG	TCT
		I									G					

Ser	Glu	Ser	Pro	Ile	Arg	Ile	Ser	Val	Ser	Thr	Glu	Gly	Thr	Asn	Thr
TCA	GAG	TCT	CCC	ATT	AGA	ATA	TCA	GTA	TCA	ACA	GAA	GGA	ACA	AAT	ACT
TCA	GAG	TCT	CCC	ATT	AGA	ATA	TCA	GTA	TCC	ACA	GAA	GGA	GCA	AAT	ACT

Ser	Ser	Ser
TCT	TCA	T
TCT	TCA	T

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47

95

102

**FIG. 31G**

CODING SEGMENT C: (SEQ ID NO: 160 (bovine) and 177 (human))

Thr	Ser	Thr	Ser	Thr	Ala	Gly	Thr	Ser	His	Leu	Val	Lys	Cys	Ala		
CC	ACA	TCC	ACA	TCT	ACA	GCT	GGG	ACA	AGC	CAT	CTT	GTC	AAG	TGT	GCA	47
I																
CT	ACA	TCT	ACA	TCC	ACC	ACT	GGG	ACA	AGC	CAT	CTT	GTA	AAA	TGT	GCG	
						T										
Glu	Lys	Glu	Lys	Thr	Phe	Cys	Val	Asn	Gly	Gly	Glu	Cys	Phe	Met	Val	
GAG	AAG	GAG	GAG	AAA	ACT	TTC	TGT	GTG	AAT	GGA	GGC	GAG	TGC	TTC	ATG	95
GAG	AAG	GAG	GAG	AAA	ACT	TTC	TGT	GTG	AAT	GGA	GGG	GAG	TGC	TTC	ATG	
Lys	Asp	Leu	Ser	Asn	Pro	Ser	Arg	Tyr	Leu	Cys						
AAA	GAC	CTT	TCA	AAT	CCC	TCA	AGA	TAC	TTG	TGC						128
AAA	GAC	CTT	TCA	AAC	CCC	TCG	AGA	TAC	TTG	TGC						

**FIG. 31H**

CODING SEGMENT C/D: (SEQ ID NO: 142 (bovine) and 178 (human))

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48

Lys	Cys	Gln	Pro	Gly	Phe	Thr	Gly	Ala	Arg	Cys	Thr	Glu	Asn	Val	Pro
AAAG	TGC	CAA	CCT	GGA	TTC	ACT	GGA	GCG	AGA	TGT	ACT	GAG	AAT	GTG	CCC
AAAG	TGC	CAA	CCT	GGA	TTC	ACT	GGA	GCA	AGA	TGT	ACT	GAG	AAT	GTG	CCC

69

Met	Lys	Val	Gln	Thr	Gln	Glu
ATG	AAA	GTC	CAA	ACC	CAA	GAA
ATG	AAA	GTC	CAA	AAC	CAA	GAA
				N		

**FIG. 311**

CODING SEGMENT D: (SEQ ID NO: 143 (bovine) and 42 (human))

Lys	Cys	Pro	Asn	Glu	Phe	Thr	Gly	Asp	Arg	Cys	Gln	Asn	Tyr	Val	Met
AAAG	TGC	CCA	AAT	GAG	TTT	ACT	GGT	GAT	CGC	TGC	CAA	AAC	TAC	GTA	ATG
AAAG	TGC	CCA	AAT	GAG	TTT	ACT	GGT	GAT	CGC	TGC	CAA	AAC	TAC	GTA	ATG

Ala	Ser	Phe	Tyr
GCC	AGC	TTC	TAC
GCC	AGC	TTC	TAC

**FIG. 31J**

CODING SEGMENT D: (SEQ ID NO: 144 (bovine) and 180 (human))

Ser	Thr	Ser	Thr	Pro	Phe	Leu	Ser	Leu	Pro	Glu *
AGT	ACG	TCC	ACT	CCC	TTT	CTG	TCT	CTG	CCT	GAA TAG
AGT	ACG	TCC	ACT	CCC	TTT	CTG	TCT	CTG	CCT	GAA TAG

**FIG. 31K**

CODING SEGMENT D': (SEQ ID NO: 145 (bovine))

Lys His Leu Gly Ile Glu Phe Met Glu  
AAG CAT CTT GGG ATT GAA TTT ATG GAG

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CODING SEGMENT H: (SEQ ID NO: 146 (bovine) and 44 (human))

## FIG. 31L

Lys	Ala	Glu	Glu	Leu	Tyr	Gln	Lys	Arg	Val	Leu	Thr	Ile	Thr	Gly	Ile	48
AAA	GCG	GAG	GAG	CTC	TAC	CAG	AAG	AGA	GTG	CTC	ACC	ATT	ACC	GGC	ATT	
AAG	GCG	GAG	GAG	CTG	TAC	CAG	AAG	AGA	GTG	CTG	ACC	ATA	ACC	GGC	ATC	
Cys	Ile	Ala	Leu	Leu	Val	Val	Gly	Ile	Met	Cys	Val	Val	Val	Tyr	Cys	96
TGC	ATC	GCG	CTG	CTC	GTG	GTT	GGC	ATC	ATG	TGT	GTG	GTG	GTC	TAC	TGC	
TGC	ATC	GCC	CTC	CTT	GTG	GTC	GGC	ATC	ATG	TGT	GTG	GTG	GCC	TAC	TGC	
Lys	Thr	Lys	Lys	Gln	Arg	Lys	Lys	Leu	His	Asp	Arg	Leu	Arg	Gln	Ser	144
AAA	ACC	AAG	AAA	CAA	CGG	AAA	AAG	CTT	CAT	GAC	CGG	CTT	CGG	CAG	AGC	
AAA	ACC	AAG	AAA	CAG	CGG	AAA	AAG	CTG	CAT	GAC	CGT	CTT	CGG	CAG	AGC	
Leu	Arg	Ser	Glu	Arg	Asn	Thr	Met	Met	Asn	Val	Ala	Asn	Gly	Pro	His	192
CTT	CGG	TCT	GAA	AGA	AAC	ACC	ATG	ATG	AAC	GTA	GCC	AAC	GGG	CCC	CAC	
CTT	CGG	TCT	GAA	CGA	AAC	AAT	ATG	ATG	AAC	ATT	GCC	AAT	GGG	CCT	CAC	
His	Pro	Asn	Pro	Pro	Pro	Glu	Asn	Val	Gln	Leu	Val	Asn	Gln	Tyr	Val	240
CAC	CCC	AAT	CCG	CCC	CCC	GAG	AAC	GTG	CAG	CTG	GTG	AAT	CAA	TAC	GTA	
CAT	CCT	AAC	CCA	CCC	CCC	GAG	AAT	GTC	CAG	CTG	GTG	AAT	CAA	TAC	GTA	
Ser	Lys	Asn	Val	Ile	Ser	Ser	Glu	His	Ile	Val	Glu	Arg	Glu	Ala	Glu	288
TCT	AAA	AAT	GTC	ATC	TCT	AGC	GAG	CAT	ATT	GTT	GAG	AGA	GAG	GCG	GAG	
TCT	AAA	AAC	GTC	ATC	TCC	AGT	GAG	CAT	ATT	GTT	GAG	AGA	GAA	GCA	GAG	

## FIG. 31L'

Ser	Ser	Phe	Ser	Thr	Ser	His	Tyr	Thr	Ser	Thr	Ala	His	His	Ser	Thr	336
AGC	TCT	TTT	TCC	ACC	AGT	CAC	TAC	ACT	TCG	ACA	GCT	CAT	CAT	TCC	ACT	
ACA	TCC	TTT	TCC	ACC	AGT	CAC	TAT	ACT	TCC	ACA	GCC	CAT	CAC	TCC	ACT	
T																
Thr	Val	Thr	Gln	Thr	Pro	Ser	His	Ser	Trp	Ser	Asn	Gly	His	Thr	Glu	384
ACT	GTC	ACT	CAG	ACT	CCC	AGT	CAC	AGC	TGG	AGC	AAT	GGA	CAC	ACT	GAA	
ACT	GTC	ACC	CAG	ACT	CCT	AGC	CAC	AGC	TGG	AGC	AAC	GGA	CAC	ACT	GAA	
Ser	Ile	Ile	Ser	Glu	Ser	His	Ser	Val	Ile	Val	Met	Ser	Ser	Val	Glu	432
AGC	ATC	ATT	TCG	GAA	AGC	CAC	TCT	GTC	ATC	GTG	ATG	TCA	TCC	GTA	GAA	
AGC	ATC	CTT	TCC	GAA	AGC	CAC	TCT	GTA	ATC	GTG	ATG	TCA	TCC	GTA	GAA	
L																
Asn	Ser	Arg	His	Ser	Pro	Ser	Thr	Gly	Gly	Pro	Arg	Gly	Arg	Leu	Asn	480
AAC	AGT	AGG	CAC	AGC	AGC	CCG	ACT	GGG	GGC	CCG	AGA	GGA	CGT	CTC	AAT	
AAC	AGT	AGG	CAC	AGC	AGC	CCA	ACT	GGG	GGC	CCA	AGA	GGA	CGT	CTT	AAT	
Gly	Leu	Gly	Gly	Pro	Arg	Glu	Cys	Asn	Ser	Phe	Leu	Arg	His	Ala	Arg	528
GGC	TTG	GGA	GGC	CCT	CGT	GAA	TGT	AAC	AGC	TTC	CTC	AGG	CAT	GCC	AGA	
GGC	ACA	GGA	GGC	CCT	CGT	GAA	TGT	AAC	AGC	TTC	CTC	AGG	CAT	GCC	AGA	
T																
Glu	Thr	Pro	Asp	Ser	Tyr	Arg	Asp	Ser	Pro	His	Ser	Glu	Arg			569
GAA	ACC	CCT	GAC	TCC	TAC	CGA	GAC	TCT	CCT	CAT	AGT	GAA	AG			
GAA	ACC	CCT	GAT	TCC	TAC	CGA	GAC	TCT	CCT	CAT	AGT	GAA	AG			

# FIG. 31M

CODING SEGMENT K: (SEQ ID NO: 161)

A	CAT	AAC	CTT	ATA	GCT	GAG	CTA	AGG	AGA	AAC	AAG	GCC	CAC	AGA	TCC	46
	His	Asn	Leu	Ile	Ala	Glu	Leu	Arg	Arg	Asn	Lys	Ala	His	Arg	Ser	
AAA	TGC	ATG	CAG	ATC	CAG	CTT	TCC	GCA	ACT	CAT	CTT	AGA	GCT	TCT	TCC	94
	Lys	Cys	Met	Gln	Ile	Gln	Leu	Ser	Ala	Thr	His	Leu	Arg	Ala	Ser	
ATT	CCC	CAT	TGG	GCT	TCA	TTC	TCT	AAG	ACC	CCT	TGG	CCT	TTA	GGA	AG	141
	Ile	Pro	His	Trp	Ala	Ser	Phe	Ser	Lys	Thr	Pro	Trp	Pro	Leu	Gly	Arg

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CODING SEGMENT L: (SEQ ID NO: 147 (bovine) and 77 (human))

	Tyr	Val	Ser	Ala	Met	Thr	Thr	Pro	Ala	Arg	Met	Ser	Pro	Val	Asp
G	TAT	GTA	TCA	GCA	ATG	ACC	ACC	CCG	GCT	CGT	ATG	TCA	CCT	GTA	GAT
I	III	II	III	II	III	III	III	III	III	III	III	III	III	III	III
G	TAT	GTG	TCA	GCC	ATG	ACC	ACC	CCG	GCT	CGT	ATG	TCA	CCT	GTA	GAT

	Phe	His	Thr	Pro	Ser	Ser	Pro	Lys	Ser	Pro	Pro	Pro	Ser	Glu	Met	Ser	Pro
TTTC	CAC	CAC	ACG	CCA	AGC	TCC	CCC	AAG	TCA	CCC	CCT	TCG	GAA	ATG	TCC	CCG	
TTTT																	
TTTC	CAC	CAC	ACG	CCA	AGC	TCC	CCC	AAA	TCG	CCC	CCT	TCG	GAA	ATG	TCT	CCA	

Pro	Val	Ser	Ser	Thr	Thr	Val	Ser	Ser	Met	Pro	Ser	Met	Ala	Val	Ser	Pro
CCC	GTG	TCC	AGC	ACG	ACG	GTC	TCC	ATG	CCC	TCC	ATG	ATG	GCG	GTC	AGT	CCC
CCC	GTG	TCC	AGC	ATG	ACG	GTG	TCC	ATG	ATG	CCT	TCC	ATG	GCG	GTC	AGC	CCC

Phe	Val	Glu	Glu	Glu	Glu	Arg	Pro	Leu	Leu	Val	Thr	Pro	Pro	Arg	Leu
TTT	GTG	GAA	GAG	GAG	GAG	AGA	CCC	CTG	CTC	CTT	GTG	ACG	CCA	CGA	CTG
TTT	ATG	GAA	GAA	GAG	GAG	AGA	CCT	CTA	CTT	CTC	GTG	ACA	CCA	CGA	CTG
N															

Arg	Glu	Lys	-	Tyr	Asp	His	His	Ala	Gln	Gln	Phe	Asn	Ser	Phe	His
CCGG	GAG	AAG	...	TAT	GAC	CAC	CAC	GCC	CAG	CAA	TTC	AAC	TCG	TTC	CAC
CCGG	GAG	AAG	AAG	TTT	GAC	CAT	CAC	CCT	CAG	CAG	TTC	AGC	TCC	TTC	CAC
			K	F				P							

Cys	Asn	Pro	Ala	His	Glu	Ser	Asn	Ser	Leu	Pro	Pro	Ser	Pro	Leu	Arg
TGC	AAC	CCC	GCG	CAT	GAG	AGC	AAC	AGC	CTG	CCC	CCC	AGC	CCC	TTG	AGG
EAC	AAC	CCC	GCG	CAT	GAC	AGT	AAC	AGC	CTC	CCT	GCT	AGC	CCC	TTG	AGG
				D							A				



**FIG. 31N:**

Ile	Val	Glu	Glu	Asp	Glu	Glu	Tyr	Glu	Thr	Thr	Gln	Glu	Tyr	Glu	Pro	Ala	334
ATA	GTG	GAG	GAT	GAG	GAA	TAT	GAA	ACG	ACC	CAG	GAG	TAC	GAA	CCA	GCT		
ATA	GTG	GAG	GAT	GAG	GAG	TAT	GAA	ACG	ACC	CAA	GAG	TAC	GAG	CCA	GCC		
Gln	Glu	Pro	Val	Lys	Lys	Leu	Thr	Asn	Ser	Ser	Arg	Arg	Ala	Lys	Arg	382	
CAA	GAG	CCG	GTT	AAG	AAA	CTC	ACC	AAC	AGC	AGC	CGG	CGG	GCC	AAA	AGA		
CAA	GAG	CCT	GTT	AAG	AAA	CTC	GCC	AA <sup>A</sup>	..T	AGC	CGG	CGG	GCC	AAA	AGA		
Thr	Lys	Pro	Asn	Gly	His	Ile	Ala	His	Arg	Leu	Glu	Met	Asp	Asn	Asn	430	
ACC	AAG	CCC	AAT	GGT	CAC	ATT	GCC	CAC	AGG	TTG	GAA	ATG	GAC	AAC	AAC		
AAC	AAG	CCC	AAT	GGC	CAC	ATT	GCT	AAC	AGA	TTG	GAA	GTG	GAC	AGC	AAC <sup>S</sup>		
Thr	Gly	Ala	Asp	Ser	Ser	Asn	Ser	Glu	Ser	Glu	Thr	Glu	Asp	Glu	Arg	478	
ACA	GGC	GCT	GAC	AGC	AGT	AAC	TCA	GAG	AGC	GAA	ACA	GAG	GAT	GAA	AGA		
ACA	AGC	TCC	CAG	AGC	AGT	AAC	TCA	GAG	AGT	GAA	ACA	GAA	GAT	GAA	AGA <sup>S</sup>		

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## FIG. 31N"

Val Gly Glu Asp Thr Pro Phe Leu Ala Ile Gln Asn Pro Leu Ala Ala	526
GTA GGA GAA GAT ACG CCT TTC CTG GCC ATA CAG AAC CCC CTG GCA GCC	
GTA GGT GAA GAT ACG CCT TTC CTG GGC ATA CAG AAC CCC CTG GCA GCC	
Ser Leu Glu Ala Ala Pro Ala Phe Arg Leu Val Asp Ser Arg Thr Asn	574
AGT CTC GAG GCG GCC CCT GGC CCT TTC CGC CTG GTC GAC AGC AGG ACT AAC	
AGT CTT GAG GCA ACA CCT GGC TTC CGC CTG GCT GAC AGC AGG ACT AAC	
Pro Thr Gly Gly Phe Ser Pro Gln Glu Glu Leu Gln Ala Arg Leu Ser	622
CCA ACA GGC GGC TTC TCT CCG CAG GAA GAA TTT CAG GCC AGG CTC TCC	
CCA GCA GGC CGC TTC TCG ACA CAG GAA GAA ATC CAG GCC AGG CTG TCT	
Gly Val Ile Ala Asn Gln Asp Pro Ile Ala Val *	672
GGT GTA ATC GCT AAC CAA GAC CCT ATC GCT GTC TAA AAC CGA AAT ACA	
AGT GTA ATT GCT AAC CAA GAC CCT ATT GCT GTA TAA AAC CTA AAT AAA	
CCC ATA GAT TCA CCT GTA AAA CTT TAT TTT ATA TAA TAA AGT ATT CCA	718
CAC ATA GAT TCA CCT GTA AAA CTT TAT TTT ATA TAA TAA AGT ATT CCA	
CCT TAA ATT AAA CAA	733
CCT TAA ATT AAA CAA	

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## FIG. 310

ATG AGA TGG CGA CGC GCC CCG CGC CGC TCC GGG CGT CCC GGC CCC CGG	48
Met Arg Trp Arg Arg Ala Pro Arg Arg Ser Gly Arg Pro Gly Pro Arg	
GCC CAG CGC CCC GGC TCC GCC GCC CGC TCG TCG CCG CCG CTG CTG	96
Ala Gln Arg Pro Gly Ser Ala Ala Arg Ser Ser Pro Pro Leu Pro Leu	
CTG CCA CTA CTG CTG CTG GGG ACC GCG GCC CTG GCG CCG GGG GCG	144
Leu Pro Leu Leu Leu Leu Leu Gly Thr Ala Ala Leu Ala Pro Gly Ala	
GCG GCC GGC AAC GAG GCG GCT CCC GCG GGG GCC TCG GTG TGC TAC TCG	192
Ala Ala Gly Asn Glu Ala Ala Pro Ala Gly Ala Ser Val Cys Tyr Ser	
TCC CCG CCC AGC GTG GGA TCG GTG CAG GAG CTA GCT CAG CGC GCC GCG	240
Ser Pro Pro Ser Val Gly Ser Val Gln Glu Leu Ala Gln Arg Ala Ala	
GTG GTG ATC GAG GGA AAG GTG CAC CCG CCG CCG CAG CAG GGG GCA	288
Val Val Ile Glu Gly Lys Val His Pro Gln Arg Arg Gln Gln Gly Ala	
CTC GAC AGG AAG GCG GCG GCG GCG GAG GCA GGG GCG TGG GGC	336
Leu Asp Arg Lys Ala Ala Ala Ala Gly Glu Ala Gly Ala Trp Gly	
GGC GAT CGC GAG CCG CCA GCC GCG GCG CCA CCG GCG CTG GGG CCG CCC	384
Gly Asp Arg Glu Pro Pro Ala Ala Gly Pro Arg Ala Leu Gly Pro Pro	
GCC GAG GAG CCG CTG CTC GCC GCG AAC GGG ACC GTG CCC TCT TGG CCC	432
Ala Glu Glu Pro Leu Leu Ala Ala Asn Gly Thr Val Pro Ser Trp Pro	
ACC GCC CCG GTG CCC AGC GCC GCG GAG CCC GGG GAG GAG GCG CCC TAT	480
Thr Ala Pro Val Pro Ser Ala Gly Glu Pro Gly Glu Glu Ala Pro Tyr	
CTG GTG AAG GTG CAC His Gln Val Trp Ala Val Lys Ala Gly Leu Lys	528
Leu Val Lys Val His Gln Val Trp Ala Val Lys Ala Gly Leu Lys	
AAG GAC TCG CTG CTC ACC GTG CGC CTG GGG ACC TGG GGC CAC CCC GCC	576
Lys Asp Ser Leu Leu Thr Val Arg Leu Gly Thr Trp Gly His Pro Ala	
TTC CCC TCC TGC GGG AGG CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC	624
Phe Pro Ser Cys Gly Arg Leu Lys Lys Glu Asp Ser Arg Tyr Ile Phe Phe	
ATG GAG CCC GAC GCC AAC AGC ACC AGC CGC GCG CCG GCC TTC CGA	672
Met Glu Pro Asp Ala Asn Ser Thr Ser Arg Ala Pro Ala Phe Arg	
GCC TCT TTC CCC CCT CTG GAG ACG GGC CCG AAC CTC AAG AAG GAG GTC	720
Ala Ser Phe Pro Pro Leu Glu Thr Gly Arg Asn Leu Lys Lys Glu Val	
AGC CGG GTG CTG TGC AAG CGG TGC G	745
Ser Arg Val Leu Cys Lys Arg Cys	

HUMAN CODING SEGMENT E:  
(SEQ ID NO: 163)

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## FIG. 32A

SEQ ID NO: 148:

AGTTTCCCC	CCCAACTTGT	CGGAACCTCTG	GGCTCGCGCG	CAGGGCAGGA	GCGGAGCGGC	60
GGCGGCTGCC	CAGGCGATGC	GAGCGCGGGC	CGGACGGTAA	TCGCCTCTCC	CTCCTCGGGC	120
TGCGAGCGCG	CCGGACCGAG	GCAGCGACAG	GAGCGGACCG	CGGCGGGAAC	CGAGGACTCC	180
CCAGCGGCGC	GCCAGCAGGA	GCCACCCCGC	GAGCGTGC GA	CCGGGACGGA	GCGCCCGCCA	240
GTCCCAGGTG	GCCCCGACCG	CACGTTGCGT	CCCCCGCGTC	CCCCCGCGCG	ACAGGAGACG	300
CTCCCCCCCCA	CGCCGCGCGC	GCCTCGGCCC	GGTCGCTGGC	CCGCCTCCAC	TCCGGGGACA	360
AACTTTTCCC	GAAGCCGATC	CCAGCCCTCG	GACCCAAACT	TGTCGCGCGT	CGCCTTCGCC	420
GGGAGCCGTC	CGCGCAGAGC	GTGCACTTCT	CGGGCGAG	ATG TCG GAG CGC AGA		475
			Met Ser Glu Arg Arg			
GAA GGC AAA	GGC AAG GGG	AAG GGC GGC	AAG AAG GAC	CGA GGC TCC	GGG	523
Glu Gly Lys	Gly Lys Gly	Lys Gly Gly	Lys Lys Asp	Arg Gly Ser	Gly	
AAG AAG CCC	GTG CCC GCG	GCT GGC GGC	CCG AGC CCA	GCC TTG CCT	CCC	571
Lys Lys Pro	Val Pro Ala	Gly Gly Pro	Ser Pro Ala	Leu Pro Pro		
CGC TTG AAA	GAG ATG AAG	ATG CAG GAG	TCT GTG GCA	GGT TCC AAA	CTA	619
Arg Leu Lys	Glu Met Lys	Ser Gln Glu	Ser Val Ala	Gly Ser Lys	Leu	
GTG CTT CGG	TGC GAG ACC	AGT TCT GAA	TAC TCC TCT	CTC AAG TTC	AAG	667
Val Leu Arg	Cys Glu Thr	Ser Ser Glu	Tyr Ser Ser	Leu Lys Phe	Lys	
TGG TTC AAG	AAT GGG AGT	GAA TTA AGC	GAA AAC AAC	CAA CCA AAC		715
Trp Phe Lys	Asn Gly Ser	Glu Leu Ser	Arg Lys Asn	Lys Pro Gln	Asn	
ATC AAG ATA	CAG AAA AGG	CCG GGG AAG	TCA GAA CTT	CGC ATT AGC	AAA	763
Ile Lys Ile	Gln Lys Arg	Pro Gly Lys	Ser Glu Leu	Arg Ile Ser	Lys	
GCG TCA CTG	GCT GAT TCT	GGA GAA TAT	ATG TGC AAA	GTG ATC AGC	AAA	811
Ala Ser Leu	Ala Asp Ser	Gly Glu Tyr	Met Cys Lys	Val Ile Ser	Lys	

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## FIG. 32B

CTA GGA AAT GAC AGT GCC TCT GCC AAC ATC ACC ATT GTG GAG TCA AAC Leu Gly Asn Asp Ser Ala Ser Ala Asn Ile Thr Ile Val Glu Ser Asn	859
GAG ATC ACC ACT GGC ATG CCA GCC TCA ACT GAG ACA GCG TAT GTG TCT Glu Ile Thr Thr Gly Met Pro Ala Ser Thr Glu Thr Ala Tyr Val Ser	907
TCA GAG TCT CCC ATT AGA ATA TCA GTA TCA ACA GAA GGA ACA AAT ACT Ser Glu Ser Pro Ile Arg Ile Ser Val Ser Thr Glu Gly Thr Asn Thr	955
TCT TCA TCC ACA TCC ACA TCT ACA GCT GGG ACA AGC CAT CTT GTC AAG Ser Ser Ser Thr Ser Thr Ser Thr Ala Gly Thr Ser His Leu Val Lys	1003
TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT GGA GGC GAG TGC TTC Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn Gly Gly Glu Cys Phe	1051
ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC TTG TGC AAG TGC CCA Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr Leu Cys Lys Cys Pro	1099
AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC TTC Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser Phe	1147
TAC AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT GAA TAGGCGCATG Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro Glu	1193
CTCAGTCGGT GCCGCTTTCT TGTGCGGCA TCTCCCCCTCA GATTCAACCT AGAGCTAGAT	1253
GCGTTTACC AGGTCTAACA TTGACTGCCCT CTGCCTGTCTG CATGAGAACCA TTAACACAAAG	1313
CGATTGTATG ACTTCCCTCTG TCCGTGACTA GTGGGCTCTG AGCTACTCGT AGGTGCGTAA	1373
GGCTCCAGTG TTTCTGAAAT TGATCTTGAA TTACTGTGAT ACGACATGAT AGTCCCTCTC	1433
ACCCAGTGCA ATGACAATAA AGGCCTTGAA AAGTCTCACT TTTATTGAGA AAATAAAAAAT	1493
CGTTCACGG GACAGTCCCT CTTCTTTATA AAATGACCCT ATCCTTGAAA AGGAGGTGTG	1553
TTAAGTTGTA ACCAGTACAC ACTTGAAATG ATGGTAAGTT CGCTTCGGTT CAGAATGTGT	1613
TCTTTCTGAC AAATAAACAG AATAAAAAAA AAAAAAAAAA A	1654

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## FIG. 33A

SEQ ID NO: 149:

CAT CAN GTG TGG GCG GCG AAA GCC GGG GGC TTG AAG AAG GAC TCG CTG His Gln Val Trp Ala Ala Lys Ala Gly Gly Leu Lys Lys Asp Ser Leu	48
CTC ACC GTG CGC CTG GGC GGC TGG GGC CAC CCC GGC TTC CCC TCC TGC Leu Thr Val Arg Leu Gly Ala Trp Gly His Pro Ala Phe Pro Ser Cys	96
GGG CGC CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC GAG Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Met Glu Pro Glu	144
GCC AAC AGC AGC GGC GGC CCC GGC CGC CTT CCG AGC CTC CTT CCC CCC Ala Asn Ser Ser Gly Gly Pro Gly Arg Leu Pro Ser Leu Leu Pro Pro	192
TCT CGA GAC GGG CCG GAA CCT CAA GAA GGA GGT CAG CCG GGT GCT GTG Ser Arg Asp Gly Pro Glu Pro Gln Glu Gly Gly Pro Gly Ala Val	240
CAA CGG TGC GCC TTG CCT CCC CGC TTG AAA GAG ATG AAG AGT CAG GAG Gln Arg Cys Ala Leu Pro Pro Arg Leu Lys Glu Met Lys Ser Gln Glu	288
TCT GTG GCA GGT TCC AAA CTA GTG CTT CCG TGC GAG ACC AGT TCT GAA Ser Val Ala Gly Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu	336
TAC TCC TCT CTC AAG TTC AAG TGG TTC AAG AAT GGG AGT GAA TTA AGC Tyr Ser Ser Leu Lys Phe Lys Trp Phe Lys Asn Gly Ser Glu Leu Ser	384
CGA AAG AAC AAA CCA GAA AAC ATC AAG ATA CAG AAA AGG CCG GGG AAG Arg Lys Asn Lys Pro Glu Asn Ile Lys Ile Gln Lys Arg Pro Gly Lys	432
TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA TAT Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu Tyr	480
ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC AAC Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn	528

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## FIG. 33B

ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG ACA Ile Thr Ile Val Glu Ser Asn Ala Thr Ser Thr Ser Thr Ala Gly Thr	576
AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn	624
GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr	672
TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn	720
GTG CCC ATG AAA GTC CAA ACC CAA GAA AAG TGC CCA AAT GAG TTT ACT Val Pro Met Lys Val Gln Thr Gln Glu Lys Cys Pro Asn Glu Phe Thr	768
GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC TTC TAC AGT ACG TCC Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser Phe Tyr Ser Thr Ser	816
ACT CCC TTT CTG TCT CTG CCT GAA TAGCGCATCT CAGTCGGTGC CGCTTTCTTG Thr Pro Phe Leu Ser Leu Pro Glu	870
TTGCCGCATC TCCCCTCAGA TTCCNCCTAG AGCTAGATGC GTTTTACCAG GTCTAACATT	930
GACTGCCCTCT GCCTGTGCGA TGAGAACATT AACACAAGCG ATTGTATGAC TTCCCTCTGTC	990
CGTGACTAGT GGGCTCTGAG CTACTCGTAG GTGCGTAAGG CTCCAGTGTT TCTGAAATTG	1050
ATCTTGAATT ACTGTGATAC GACATGATAG TCCCCTCTCAC CCAGTGCAAT GACAATAAAG	1110
GCCTTGAAAA GTCAAAAAAA AAAAAAAAAA	1140

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## FIG. 34A

SEQ ID NO: 150:

G AAG TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA Lys Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu	49
TAT ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC Tyr Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala	97
AAC ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG Asn Ile Thr Ile Val Glu Ser Asn Ala Thr Ser Thr Ser Thr Ala Gly	145
ACA AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG Thr Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val	193
AAT GGA GGC GAC TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA Asn Gly Gly Asp Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg	241
TAC TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA CCG AGA TGT ACT GAG Tyr Leu Cys Lys Cys Gln Pro Gly Phe Thr Thr Gly Ala Arg Cys Thr Glu	289
AAT GTG CCC ATG AAA GTC CAA ACC CAA GAA AAA GCG GAG GAG CTC TAC Asn Val Pro Met Lys Val Gln Thr Gln Glu Lys Ala Glu Leu Tyr	337
CAG AAG AGA GTG CTC ACC ATT ACC GGC ATT TGC ATC GCG CTG CTC GTG Gln Lys Arg Val Leu Thr Ile Thr Gly Ile Cys Ile Ala Leu Val	385
GTT GGC ATC ATG TGT GTG GTG GTC TAC TGC AAA ACC AAG AAA CAA CGG Val Gly Ile Met Cys Val Val Val Tyr Cys Lys Thr Lys Lys Gln Arg	433
AAA AAG CTT CAT GAC CCG CTT CCG CAG AGC CTT CGG TCT GAA AGA AAC Lys Lys Leu His Asp Arg Leu Arg Gln Ser Leu Arg Ser Glu Arg Asn	481
ACC ATG ATG AAC GTA GCC AAC GGG CCC CAC CAC CCC AAT CCG CCC CCC Thr Met Met Asn Val Ala Asn Gly Pro His His Pro Asn Pro Pro Pro	529
GAG AAC GTG CAG CTG GTG AAT CAA TAC GTA TCT AAA AAT GTC ATC TCT Glu Asn Val Gln Leu Val Asn Gln Tyr Val Ser Lys Asn Val Ile Ser	577



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## FIG. 34B

AGC GAG CAT ATT GTT GAG AGA GAG GCG GAG AGC TCT TTT TCC ACC AGT Ser Glu His Ile Val Glu Arg Glu Ala Glu Ser Ser Phe Ser Thr Ser	625
CAC TAC ACT TCG ACA GCT CAT CAT CAT TCC ACT ACT GTC ACT CAG ACT CCC His Tyr Thr Ser Thr Ala His His Ser Thr Thr Val Thr Gln Thr Pro	673
AGT CAC AGC TGG AGC AAT GGA CAC ACT GAA AGC ATC ATT TCG GAA AGC Ser His Ser Trp Ser Ser Asn Gly His Thr Glu Ser Ile Ile Ser Glu Ser	721
CAC TCT GTC ATC GTG ATG TCA TCC GTA GAA AAC AGT AGG CAC AGC AGC His Ser Val Ile Val Met Ser Ser Val Glu Asn Ser Arg His Ser Ser	769
CCG ACT GGG GGC CCG AGA GGA CGT CTC AAT GGC TTG GGA GGC CCT CGT Pro Thr Gly Gly Pro Arg Arg Gly Arg Leu Asn Gly Leu Gly Pro Arg	817
GAA TGT AAC AGC TTC CTC AGG CAT GCC AGA GAA ACC CCT GAC TCC TAC Glu Cys Asn Ser Phe Leu Arg His Ala Arg Glu Thr Pro Asp Ser Tyr	865
CGA GAC TCT CCT CAT AGT GAA AGA CAT AAC CTT ATA GCT GAG CTA AGG Arg Asp Ser Pro His Ser Ser Glu Arg His Asn Leu Ile Ala Glu Leu Arg	913
AGA AAC AAG GCC CAC AGA TCC AAA TGC ATG CAG ATC CAG CTT TCC GCA Arg Asn Lys Ala His Arg Ser Lys Cys Met Gln Ile Gln Leu Ser Ala	961
ACT CAT CTT AGA GCT TCT TCC TCC ATT CCC CAT TGG GCT TCA TTC TCT AAG Thr His Leu Arg Ala Ser Ser Ile Pro His Trp Ala Ser Phe Ser Lys	1009
ACC CCT TGG CCT TTA GGA AGG TAT GTA TCA GCA ATG ACC ACC CCG GCT Thr Pro Trp Pro Leu Gly Arg Tyr Val Ser Ala Met Thr Thr Pro Ala	1057
CGT ATG TCA CCT GTA GAT TTC CAC ACG CCA AGC TCC CCC AAG TCA CCC Arg Met Ser Pro Val Asp Phe His Thr Pro Ser Ser Pro Lys Ser Pro	1105
CCT TCG GAA ATG TCC CCG CCC GTG TCC AGC ACG GTC TCC ATG CCC Pro Ser Glu Met Ser Pro Pro Val Ser Ser Thr Thr Val Ser Met Pro	1153

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## FIG. 34C

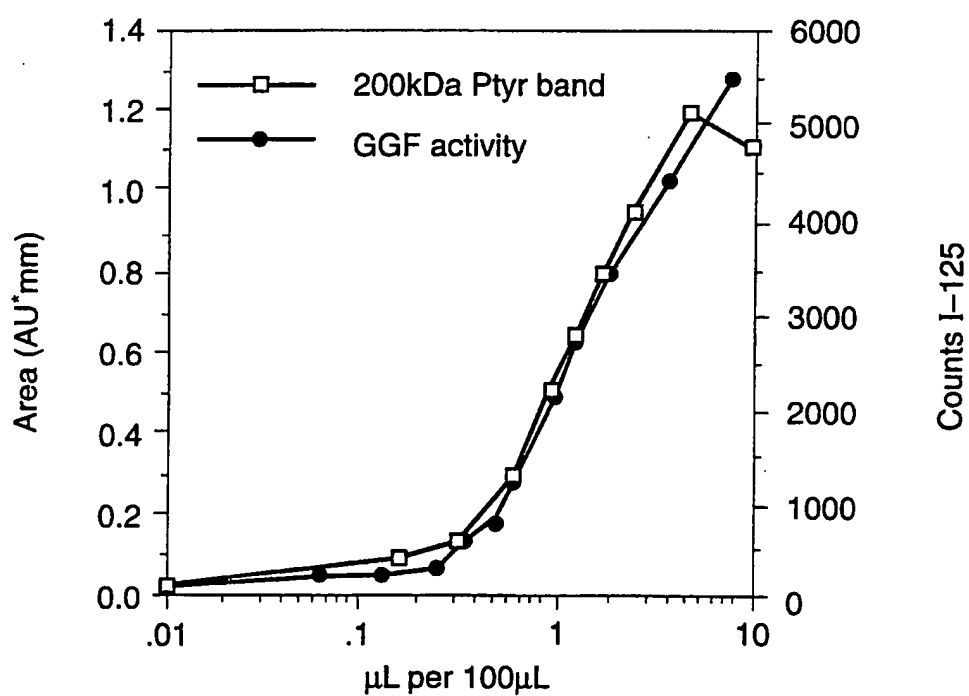
TCC ATG GCG GTC AGT CCC TTC GTG GAA GAG GAG AGA CCC CTG CTC CTT	1201
Ser Met Ala Val Ser Pro Phe Val Glu Glu Glu Arg Pro Leu Leu	
GTG ACG CCA CCA CCG CTG CCG GAG AAG TAT GAC CAC CAC GCC CAG CAA	1249
Val Thr Pro Pro Arg Leu Arg Glu Lys Tyr Asp His His Ala Gln Gln	
TTC AAC TCG TTC CAC TGC AAC CCC GCG CAT GAG AGC AAC AGC CTG CCC	1297
Phe Asn Ser Phe His Cys Asn Pro Ala His Glu Ser Asn Ser Leu Pro	
CCC AGC CCC TTG AGG ATA GTG GAG GAT GAG GAA TAT GAA ACG ACC CAG	1345
Pro Ser Pro Leu Arg Ile Val Glu Asp Glu Glu Tyr Glu Thr Thr Gln	
GAG TAC GAA CCA GCT CAA GAG CCG GTT AAG AAA CTC ACC AAC AGC AGC	1393
Glu Tyr Glu Pro Ala Gln Glu Pro Val Lys Lys Leu Thr Asn Ser Ser	
CGG CGG GCC AAA AGA ACC AAG CCC AAT GGT CAC ATT GCC CAC AGG TTG	1441
Arg Arg Ala Lys Arg Thr Lys Pro Asn Gly His Ile Ala His Arg Leu	
GAA ATG GAC AAC AAC ACA GGC GCT GAC AGC AGT AAC TCA GAG AGC GAA	1489
Glu Met Asp Asn Asn Thr Gly Ala Asp Ser Ser Asn Ser Glu Ser Glu	
ACA GAG GAT GAA AGA GTA GGA GAA GAT ACG CCT TTC CTG GCC ATA CAG	1537
Thr Glu Asp Glu Arg Val Gly Glu Asp Thr Pro Phe Leu Ala Ile Gln	
AAC CCC CTG GCA GCC AGT CTC GAG GCG GCC CCT GCC TTC CGC CTG GTC	1585
Asn Pro Leu Ala Ala Ser Leu Glu Ala Ala Pro Ala Phe Arg Leu Val	
GAC AGC AGG ACT AAC CCA ACA GGC GGC TTC TCT CCG CAG GAA GAA TTG	1633
Asp Ser Arg Thr Asn Pro Thr Gly Gly Phe Ser Pro Gln Glu Glu Leu	
CAG GCC AGG CTC TCC GGT GTA ATC GCT AAC CAA GAC CCT ATC GCT GTC	1681
Gln Ala Arg Leu Ser Gly Val Ile Ala Asn Gln Asp Pro Ile Ala Val	
TAAAACCGAA ATACACCCCAT AGATTACCT GTAAAACCTT ATTTTATATA ATAAAGTATT	1741
CCACCTTAAA TTAAACAAAA AAA	1764

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# FIG. 35

GGF2bpp5	(SEQ ID NO: 151)	KCAEKEKTF	CVNGGECF	MVKDLS	NP	SRYLCK	CPNEFT	GDRCQ	NYVMASFY	
										*
GGF2bpp4	(SEQ ID NO: 152)	KCAEKEKTF	CVNGGDCF	MVKDLS	NP	SRYLCK	CPGFT	GARCTEN	VPMKVQ	
hEGF	(SEQ ID NO: 153)	ECLRKYKDF	CIH-GECKYV	KELRAPS---	CKCQ	QEQYF	GERC	GEKSNK	THS	

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**FIG. 36**

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**FIG. 37A**

F-B-A'

F-B-A-C-C/D-D  
 F-B-A-C-C/D-H  
 F-B-A-C-C/D-H-L  
 F-B-A-C-C/D-H-K-L  
 F-B-A-C-C/D-D'-H  
 F-B-A-C-C/D-D'-H-L  
 F-B-A-C-C/D-D'-H-K-L  
 F-B-A-C-C/D'-D  
 F-B-A-C-C/D'-H  
 F-B-A-C-C/D'-H-L  
 F-B-A-C-C/D'-H-K-L  
 F-B-A-C-C/D'-D'-H  
 F-B-A-C-C/D'-D'-H-L  
 F-B-A-C-C/D'-D'-H-K-L  
 F-B-A-C-C/D-C/D'-D  
 F-B-A-C-C/D-C/D'-H  
 F-B-A-C-C/D-C/D'-H-L  
 F-B-A-C-C/D-C/D'-H-K-L  
 F-B-A-C-C/D-C/D'-D'-H  
 F-B-A-C-C/D-C/D'-D'-H-L  
 F-B-A-C-C/D-C/D'-D'-H-K-L

F-B-A-G-C-C/D-D  
 F-B-A-G-C-C/D-H  
 F-B-A-G-C-C/D-H-L  
 F-B-A-G-C-C/D-H-K-L  
 F-B-A-G-C-C/D-D'-H  
 F-B-A-G-C-C/D-D'-H-L  
 F-B-A-G-C-C/D-D'-H-K-L  
 F-B-A-G-C-C/D'-D  
 F-B-A-G-C-C/D'-H  
 F-B-A-G-C-C/D'-H-L  
 F-B-A-G-C-C/D'-H-K-L  
 F-B-A-G-C-C/D'-D'-H  
 F-B-A-G-C-C/D'-D'-H-L  
 F-B-A-G-C-C/D'-D'-H-K-L  
 F-B-A-G-C-C/D-C/D'-D  
 F-B-A-G-C-C/D-C/D'-H  
 F-B-A-G-C-C/D-C/D'-H-L  
 F-B-A-G-C-C/D-C/D'-H-K-L  
 F-B-A-G-C-C/D-C/D'-D'-H  
 F-B-A-G-C-C/D-C/D'-D'-H-L  
 F-B-A-G-C-C/D-C/D'-D'-H-K-L

F-E-B-A'

F-E-B-A-C-C/D-D  
 F-E-B-A-C-C/D-H  
 F-E-B-A-C-C/D-H-L  
 F-E-B-A-C-C/D-H-K-L  
 F-E-B-A-C-C/D-D'-H  
 F-E-B-A-C-C/D-D'-H-L  
 F-E-B-A-C-C/D-D'-H-K-L  
 F-E-B-A-C-C/D'-D  
 F-E-B-A-C-C/D'-H  
 F-E-B-A-C-C/D'-H-L  
 F-E-B-A-C-C/D'-H-K-L  
 F-E-B-A-C-C/D'-D'-H  
 F-E-B-A-C-C/D'-D'-H-L  
 F-E-B-A-C-C/D'-D'-H-K-L  
 F-E-B-A-C-C/D-C/D'-D  
 F-E-B-A-C-C/D-C/D'-H  
 F-E-B-A-C-C/D-C/D'-H-L  
 F-E-B-A-C-C/D-C/D'-H-K-L  
 F-E-B-A-C-C/D-C/D'-D'-H  
 F-E-B-A-C-C/D-C/D'-D'-H-L  
 F-E-B-A-C-C/D-C/D'-D'-H-K-L

F-E-B-A-G-C-C/D-D  
 F-E-B-A-G-C-C/D-H  
 F-E-B-A-G-C-C/D-H-L  
 F-E-B-A-G-C-C/D-H-K-L  
 F-E-B-A-G-C-C/D-D'-H  
 F-E-B-A-G-C-C/D-D'-H-L  
 F-E-B-A-G-C-C/D-D'-H-K-L  
 F-E-B-A-G-C-C/D'-D  
 F-E-B-A-G-C-C/D'-H  
 F-E-B-A-G-C-C/D'-H-L  
 F-E-B-A-G-C-C/D'-H-K-L  
 F-E-B-A-G-C-C/D'-D'-H  
 F-E-B-A-G-C-C/D'-D'-H-L  
 F-E-B-A-G-C-C/D'-D'-H-K-L  
 F-E-B-A-G-C-C/D-C/D'-D  
 F-E-B-A-G-C-C/D-C/D'-H  
 F-E-B-A-G-C-C/D-C/D'-H-L  
 F-E-B-A-G-C-C/D-C/D'-H-K-L  
 F-E-B-A-G-C-C/D-C/D'-D'-H  
 F-E-B-A-G-C-C/D-C/D'-D'-H-L  
 F-E-B-A-G-C-C/D-C/D'-D'-H-K-L

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**FIG. 37B**

E-B-A'

E-B-A-C-C/D-D  
 E-B-A-C-C/D-H  
 E-B-A-C-C/D-H-L  
 E-B-A-C-C/D-H-K-L  
 E-B-A-C-C/D-D'-H  
 E-B-A-C-C/D-D'-H-L  
 E-B-A-C-C/D-D'-H-K-L  
 E-B-A-C-C/D'-D  
 E-B-A-C-C/D'-H  
 E-B-A-C-C/D'-H-L  
 E-B-A-C-C/D'-H-K-L  
 E-B-A-C-C/D'-D'-H  
 E-B-A-C-C/D'-D'-H-L  
 E-B-A-C-C/D'-D'-H-K-L  
 E-B-A-C-C/D-C/D'-D  
 E-B-A-C-C/D-C/D'-H  
 E-B-A-C-C/D-C/D'-H-L  
 E-B-A-C-C/D-C/D'-H-K-L  
 E-B-A-C-C/D-C/D'-D'-H  
 E-B-A-C-C/D-C/D'-D'-H-L  
 E-B-A-C-C/D-C/D'-D'-H-K-L

E-B-A-G-C-C/D-D  
 E-B-A-G-C-C/D-H  
 E-B-A-G-C-C/D-H-L  
 E-B-A-G-C-C/D-H-K-L  
 E-B-A-G-C-C/D-D'-H  
 E-B-A-G-C-C/D-D'-H-L  
 E-B-A-G-C-C/D-D'-H-K-L  
 E-B-A-G-C-C/D'-D  
 E-B-A-G-C-C/D'-H  
 E-B-A-G-C-C/D'-H-L  
 E-B-A-G-C-C/D'-H-K-L  
 E-B-A-G-C-C/D'-D'-H  
 E-B-A-G-C-C/D'-D'-H-L  
 E-B-A-G-C-C/D'-D'-H-K-L  
 E-B-A-G-C-C/D-C/D'-D  
 E-B-A-G-C-C/D-C/D'-H  
 E-B-A-G-C-C/D-C/D'-H-L  
 E-B-A-G-C-C/D-C/D'-H-K-L  
 E-B-A-G-C-C/D-C/D'-D'-H  
 E-B-A-G-C-C/D-C/D'-D'-H-L  
 E-B-A-G-C-C/D-C/D'-D'-H-K-L

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## FIG. 38

SEQ ID NO: 154:

AGC	CAT	CTT	GTC	AAG	TGT	GCA	GAG	AAG	GAG	AAA	ACT	TTC	TGT	GTG	AAT	48
Ser	His	Leu	Val	Lys	Cys	Ala	Glu	Lys	Glu	Lys	Thr	Phe	Cys	Val	Asn	
GGA	GGC	GAG	TGC	TTC	ATG	GTG	AAA	GAC	CTT	TCA	AAT	CCC	TCA	AGA	TAC	96
Gly	Gly	Glu	Cys	Phe	Met	Val	Lys	Asp	Leu	Ser	Asn	Pro	Ser	Arg	Tyr	
TTG	TGC	AAG	TGC	CCA	AAT	GAG	TTT	ACT	GGT	GAT	CGC	TGC	CAA	AAC	TAC	144
Leu	Cys	Lys	Cys	Pro	Asn	Glu	Phe	Thr	Gly	Asp	Arg	Cys	Gln	Asn	Tyr	
GTA	ATG	GCC	AGC	TTC	TAC	AGT	ACG	TCC	ACT	CCC	TTT	CTG	TCT	CTG	CCT	192
Val	Met	Ala	Ser	Phe	Tyr	Ser	Thr	Ser	Thr	Pro	Phe	Leu	Ser	Leu	Pro	
GAA	TAG															198
Glu																

## FIG. 39

SEQ ID NO: 155:

AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT	48
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn	
GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC	96
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr	
TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT	144
Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn	
GTG CCC ATG AAA GTC CAA ACC CAA GAA AAA GCG GAG GAG CTC TAC TAA	192
Val Pro Met Lys Val Gln Thr Gln Glu Lys Ala Glu Glu Leu Tyr	

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# FIG. 40

SEQ ID NO: 156:

AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT	48
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn	
GGA GGC GAG TGC TTC ATG ATG AAA GAC CTT TCA AAT CCC TCA AGA TAC	96
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr	
TTG TGC AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC	144
Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr	
GTA ATG GCC AGC TTC TAC AAA GCG GAG GAG CTC TAC TAA	183
Val Met Ala Ser Phe Tyr Lys Ala Glu Glu Leu Tyr	

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**FIG. 41**

SEQ ID NO: 157:

AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT	48
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn	
GGA GGC GAG TGC TTC ATG ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC	96
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr	
TTG TGC AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC	144
Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr	
GTA ATG GCC AGC TTC TAC AAG CAT CTT GGG ATT GAA TTT ATG GAG AAA	192
Val Met Ala Ser Phe Tyr Lys His Leu Gly Ile Glu Phe Met Glu Lys	
GCG GAG GAG CTC TAC TAA	210
Ala Glu Glu Leu Tyr	

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## FIG. 42

SEQ ID NO: 158:

AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT	48
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn	
GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC	96
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr	
TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT	144
Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn	
GTG CCC ATG AAA GTC CAA ACC CAA GAA AAG TGC CCA AAT GAG TTT ACT	192
Val Pro Met Lys Val Gln Thr Thr Gln Glu Lys Cys Pro Asn Glu Phe Thr	
GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC TTC TAC AGT ACG TCC	240
Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser Phe Tyr Ser Thr Ser	
ACT CCC TTT CTG TCT CTG CCT GAA TAG	267
Thr Pro Phe Leu Ser Leu Pro Glu	

## FIG. 43

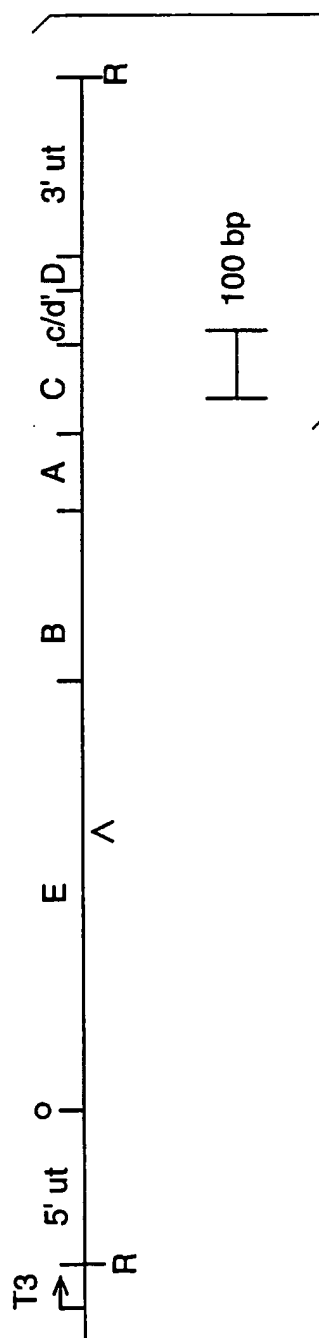
SEQ ID NO: 159:

AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT	48
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn	
GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC	96
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr	
TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT	144
Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn	
GTG CCC ATG AAA GTC CAA ACC CAA GAA AAG TGC CCA AAT GAG TTT ACT	192
Val Pro Met Lys Val Gln Thr Gln Glu Lys Cys Pro Asn Glu Phe Thr	
GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC TTC TAC AAA GCG GAG	240
Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser Phe Tyr Lys Ala Glu	
GAG CTC TAC TAA	252
Glu Leu Tyr	

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FIG. 44



## FIG. 45A

SEQ ID NO: 21:

GGAATCCCTT TTTTTTTT TTTTTTCTT NTTTTTTTTT TGCCCTTATA CCTCTCGCC 60  
 TTTCTGTGGT TCCATCCACT TCTTCCCCCT CCTCCTCCCA TAAACAACTC TCCTACCCCT 120  
 GCACCCCCAA TAAATAAATA AAAGGAGGAG GGCAAGGGG GAGGAGGAGG AGTGGTGCTG 180  
 CGAGGGGAAG GAAAAGGAG GCAGCGCGAG AAGAGCCGGG CAGAGTCCGA ACCGACAGCC 240  
 AGAAGCCCGC ACGCACCTCG CACC ATG AGA TGG CGA CGC GCC CCG CGC CGC 291  
 Met Arg Trp Arg Arg Ala Pro Arg Arg  
 TCC GGG CGT CCC GGC CCC CGG GCC CAG CGC CCC GGC TCC GCC GCC CGC 339  
 Ser Gly Arg Pro Gly Pro Arg Ala Gln Arg Pro Gly Ser Ala Ala Arg  
 TCG TCG CCG CCG CTG CCG CTG CCG CCA CTA CTG CTG CTG CTG GGG ACC 387  
 Ser Ser Pro Pro Leu Pro Leu Leu Pro Leu Leu Leu Leu Thr Val  
 Val Cys Leu Leu Thr Val GGF-II 09  
 GCG GCC CTG GCG CCG GGG GCG GCG GCC GGC AAC GAG GCG GCT CCC GCG 435  
 Ala Ala Leu Ala Pro Gly Ala Ala Ala Gly Asn Glu Ala Ala Pro Ala  
 Ala Ala Leu Pro Pro  
 GGG GCC TCG GTG TGC TAC TCG TCC CCG CCC AGC GTG GGA TCG GTG CAG 483  
 Gly Ala Ser Val Cys Tyr Ser Ser Pro Pro Ser Val Gly Ser Val Gln  
 Ala Ser Pro Val Ser Val Gly Ser Val Gln GGF-II 08  
 GAG CTA GCT CAG CGC GCC GCG GTG GTG ATC GAG GGA AAG GTG CAC CCG 531  
 Glu Leu Ala Gln Arg Ala Ala Val Val Ile Glu Gly Lys Val His Pro  
 Glu Leu Val Gln Arg Trp Phe val val Ile Glu Gly Lys  
 GGF-II 04

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**FIG. 45B**

579	CAG CGG CGG CAG GGG GCA CTC GAC AGG AAG GCG GCG GCG GCG GCG	Gln Arg Arg Gln Gln Gly Ala Leu Asp Arg Lys Ala Ala Ala Ala
627	GGC GAG GCA GGG GCG TGG GGC GGC GAT CGC GAG CCG CCA GCC GCG GGC	Gly Glu Ala Ala Gly Ala Trp Gly Gly Asp Arg Glu Pro Pro Ala Ala Gly
675	CCA CGG GCG CTG GGG CCG CCC GCG GAG GAG CCG CTG CTC GCC GCC AAC	Pro Arg Ala Leu Gly Pro Pro Ala Glu Glu Pro Leu Leu Ala Ala Asn
723	GGG ACC GTG CCC TCT TGG CCC ACC GCC CCG GTG CCC AGC GCC GGC GAG	Gly Thr Val Pro Ser Trp Pro Thr Ala Pro Val Pro Ser Ala Gly Glu
771	CCC GGG GAG GAG GCG CCC TAT CTG GTG AAG GTG CAC CAG GTG TGG GCG	Pro Gly Glu Glu Ala Pro Tyr Leu Val Lys Val His Gln Val Trp Ala
819	GTG AAA GCC GGG GGC TTG AAG AAG GAC TCG CTG CTC ACC GTG CGC CTG	Val Lys Ala Gly Gly Leu Lys Lys Asp Ser Leu Leu Thr Val Arg Leu
867	GGG ACC TGG GGC CAC CCC GCC TTC TCC TGC GGG AGG CTC AAG GAG	Gly Thr Trp Gly His Pro Ala Phe Pro Ser Cys Gly Arg Leu Lys Glu
915	GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC GAC GCC AAC AGC ACC AGC	Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro Asp Ala Asn Ser Thr Ser

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## FIG. 45C

CGC GCG CCG GCC GCC TTC CGA GCC TCT TTC CCC CCT CTG GAG ACG GGC Arg Ala Pro Ala Ala Phe Arg Ala Ser Phe Pro Pro Leu Glu Thr Gly	963
CGG AAC CTC AAG AAG GAG GTC AGC CGG GTG CTG TGC AAG CGG TGC GCC Arg Asn Leu Lys Lys Glu Val Ser Arg Val Leu Cys Lys Arg Cys Ala	1011
TTG CCT CCC CAA TTG AAA GAG ATG AAA AGC CAG GAA TCG GCT GCA GGT Leu Pro Pro Gln Leu Lys Glu Met Lys Ser Gln Glu Ser Ala Ala Gly	1059
TCC AAA CTA GTC CTT CGG TGT GAA ACC AGT TCT GAA TAC TCC TCT CTC Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu Tyr Ser Ser Leu Leu Val Leu Arg GGF-II 06	1107
AGA TTC AAG TGG TTC AAG AAT GGG AAT GAA TTG AAT CGA AAA AAC AAA Arg Phe Lys Lys Trp Phe Lys Asn Gly Asn Glu Leu Asn Arg Lys Asn Lys	1155
CCA CAA AAT ATC AAG ATA CAA AAA AAG CCA GGG AAG TCA GAA CTT CGC Pro Gln Asn Ile Lys Ile Gln Lys Lys Pro Gly Lys Ser Glu Leu Arg	1203
ATT AAC AAA GCA TCA CTG GCT GAT TCT GGA GAG TAT ATG TGC AAA GTG Ile Asn Lys Ala Ser Leu Ala Asp Ser Gly Glu Tyr Met Cys Lys Val Lys Ala Ser Leu Ala Asp Ser Gly Glu Tyr Met Xaa Lyx GGF-II 12	1251
ATC AGC AAA TTA GGA AAT GAC AGT GCC TCT GCC AAT ATC ACC ATC GTG Ile Ser Lys Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn Ile Thr Ile Val	1299
GAA TCA AAC GCT ACA TCT ACA TCC ACC ACT GGG ACA AGC CAT CTT GTA Glu Ser Asn Ala Thr Ser Thr Ser Thr Gly Thr Ser His Leu Val	1347

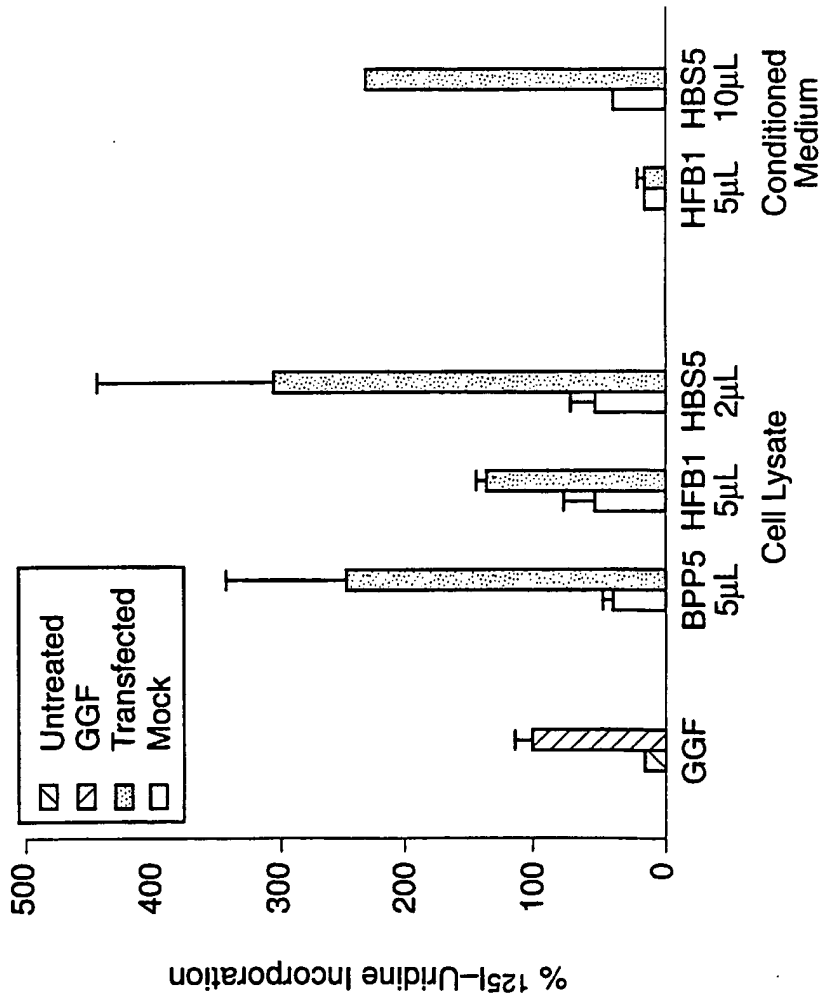


## FIG. 45D

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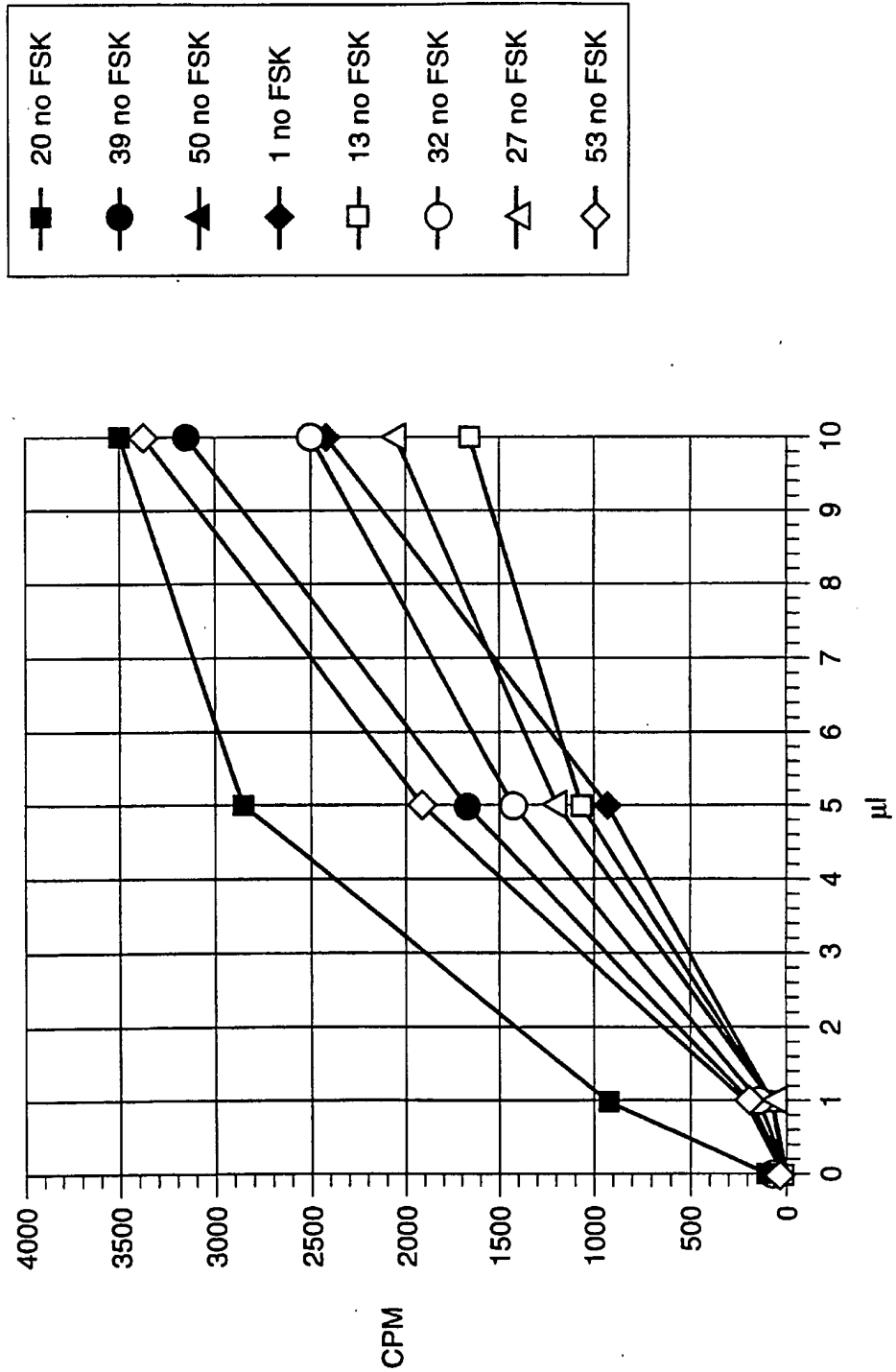
AAA TGT GCG GAG AAG GAG AAA ACT TTC TGT GTG AAT GGA GGG GAG TGC Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn Gly Gly Glu Cys	1395
TTC ATG GTG AAA GAC CTT TCA AAC CCC TCG AGA TAC TTG TGC AAG TGC Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr Leu Cys Lys Cys	1443
CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser	1491
TTC TAC AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT GAA Phe Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro Glu	1530
TAGGAGCATG CTCAGTTGGT GCTGCTTTCT TGTTCGTGCA TCTCCCTCTCA GATTCCACCT	1590
AGAGCTAGAT GTGTCTTACC AGATCTAATA TTGACTGCCT CTGCCCTGTCG CATGAGAACA	1650
TTAACAAAAAG CAATTGTATT ACTTCCTCTG TTCGCGACTA GTTGGCTCTG AGATACTAAT	1710
AGGTGTGTGA GGCTCCGGAT GTTTCCTGGAA TTGATATTGA ATGATGTGAT ACAAATTGAT	1770
AGTCAATATC AAGCAGTGAA ATATGATAAT AAAGGCATTT CAAAGTCTCA CTTTATTGA	1830
TAAAAATAAAA ATCATTTCTAC TGAACAGTCC ATCTTCTTTA TACAATGACC ACATCCTGAA	1890
AAGGTGTTG CTAAGCTGTA ACCGATATGC ACTTGAAATG ATGCTAAGTT AATTTTGATT	1950
CAGAATGTGT TATTTGTCAC AAATAAACAT AATAAAAGGA AAAAAA AAA	2003

FIG. 46



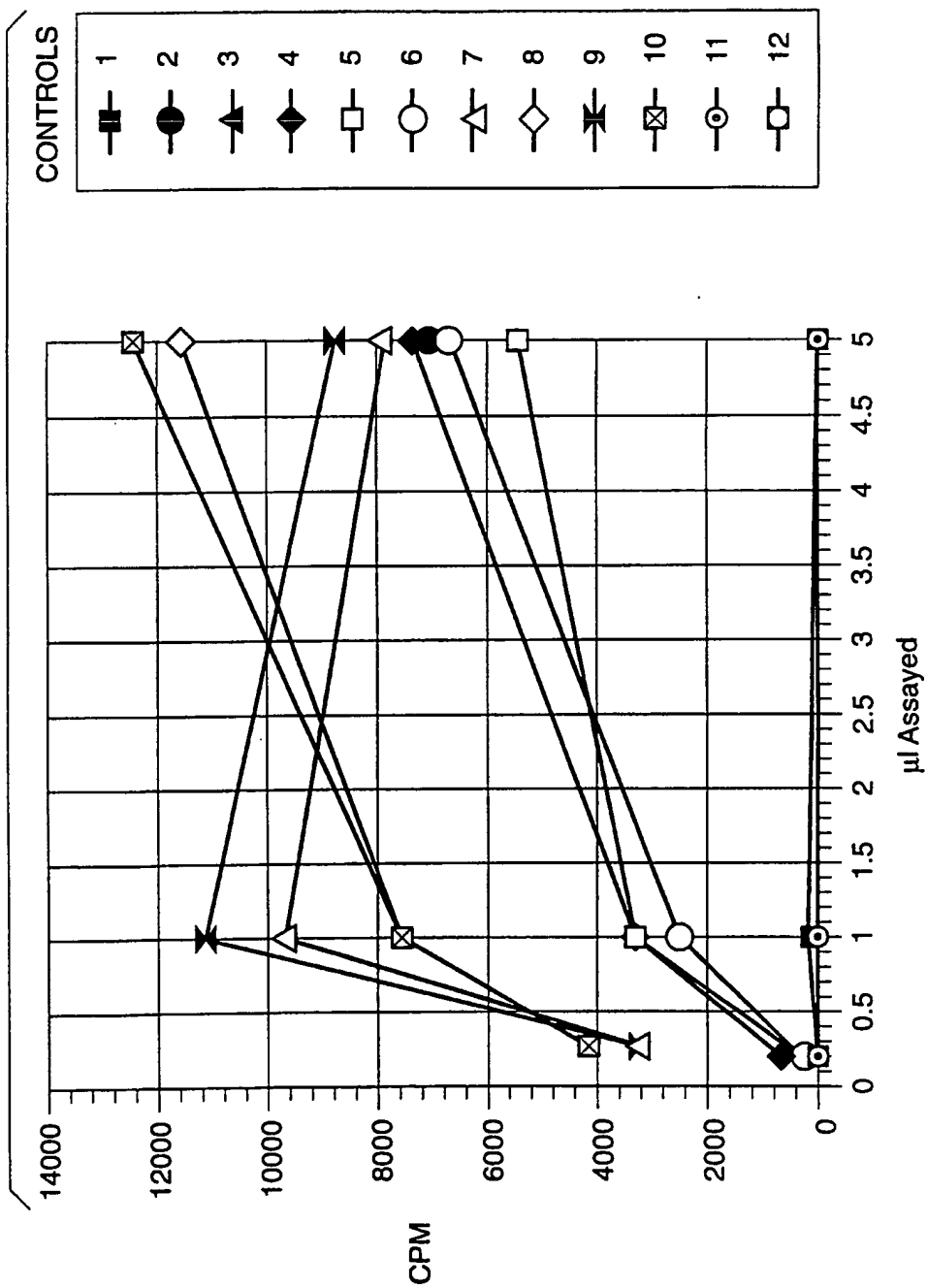
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FIG. 47



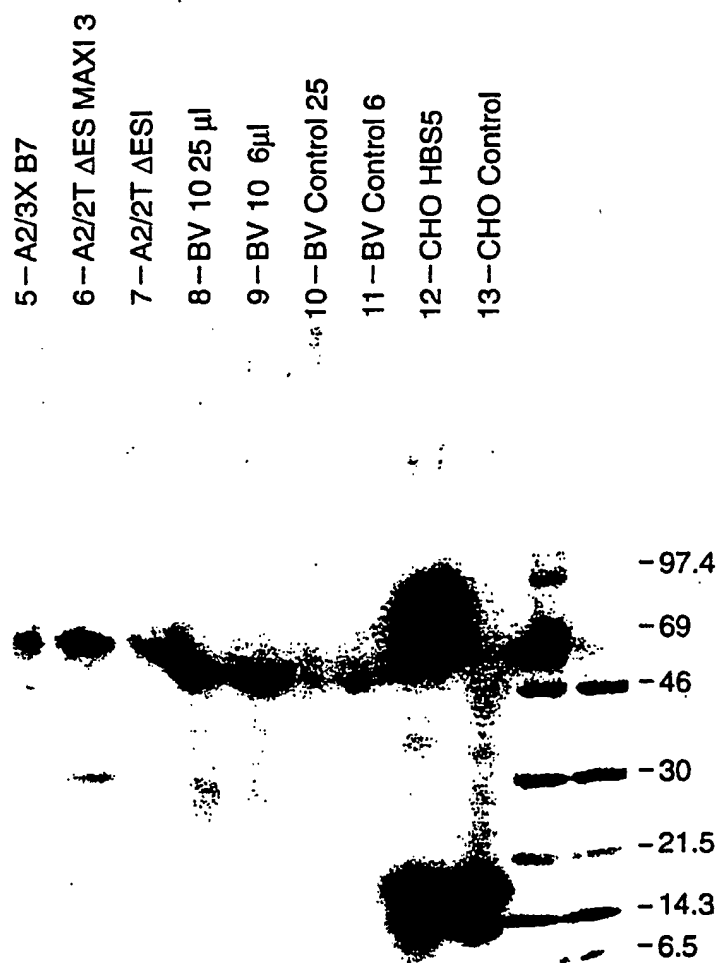
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FIG. 48



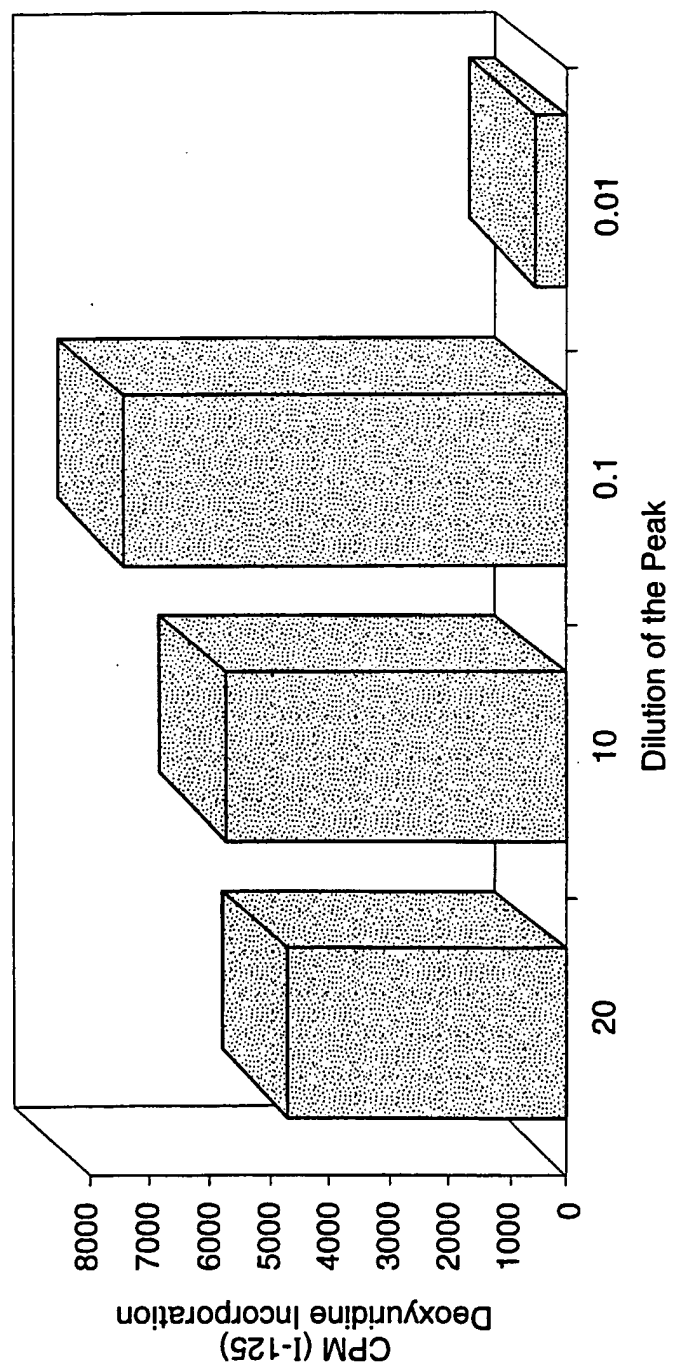
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FIG. 49



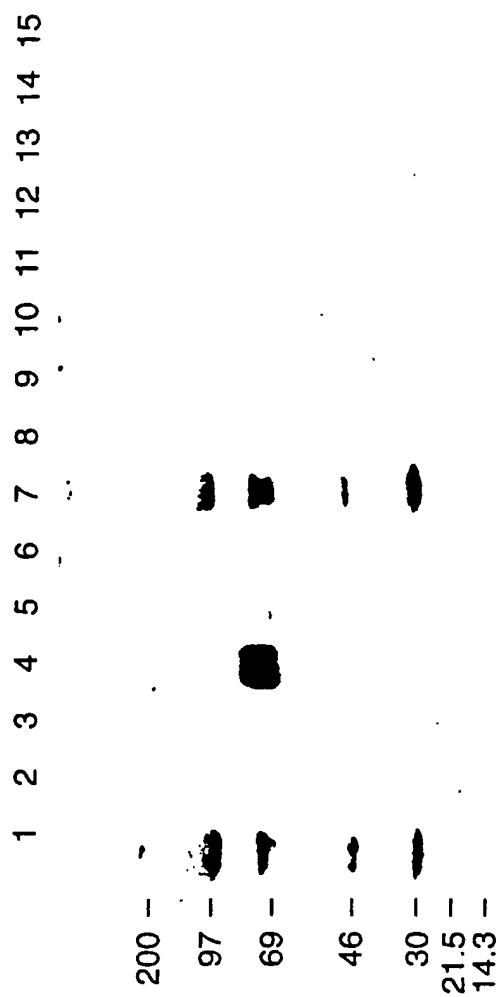
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FIG. 50A



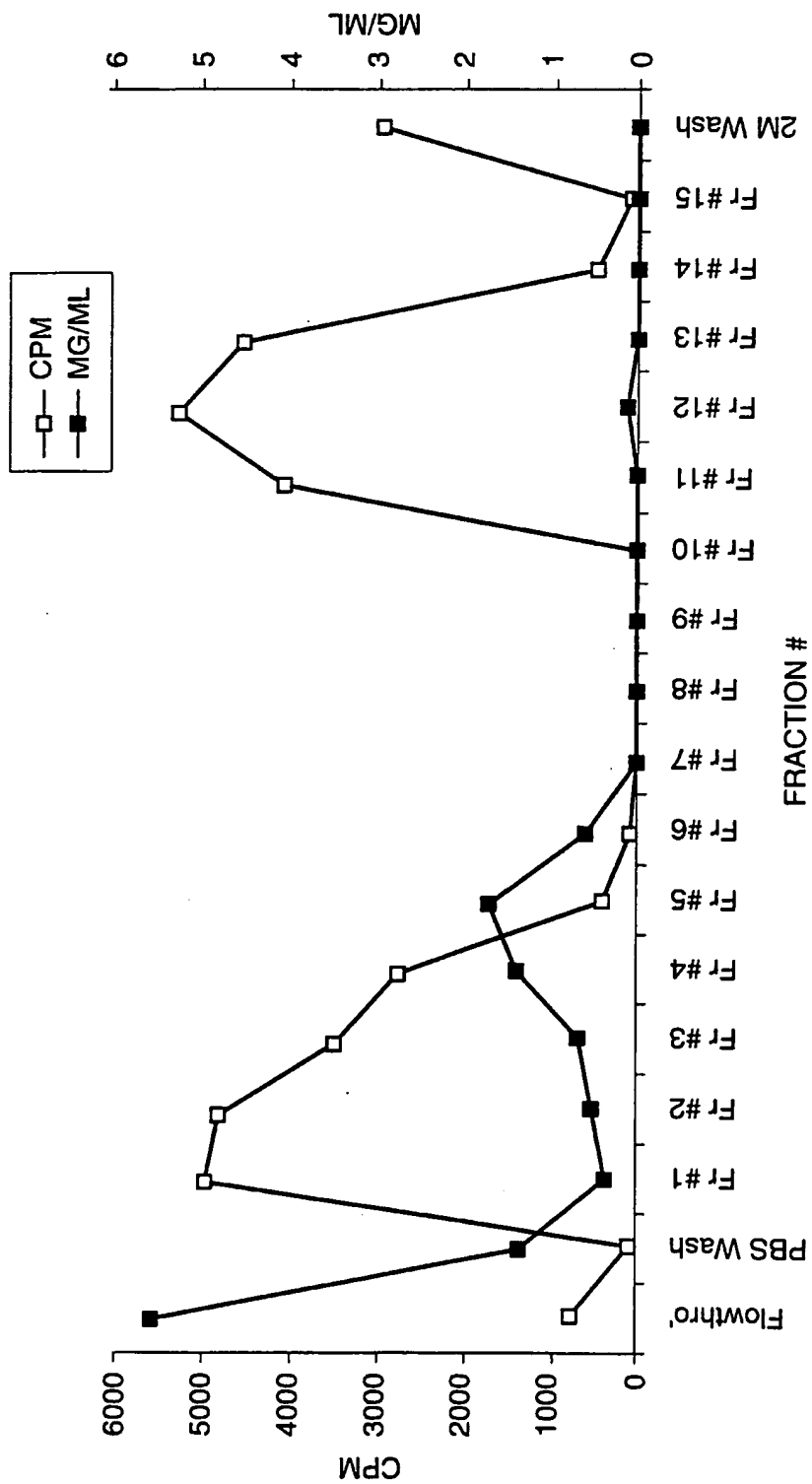
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FIG. 50B



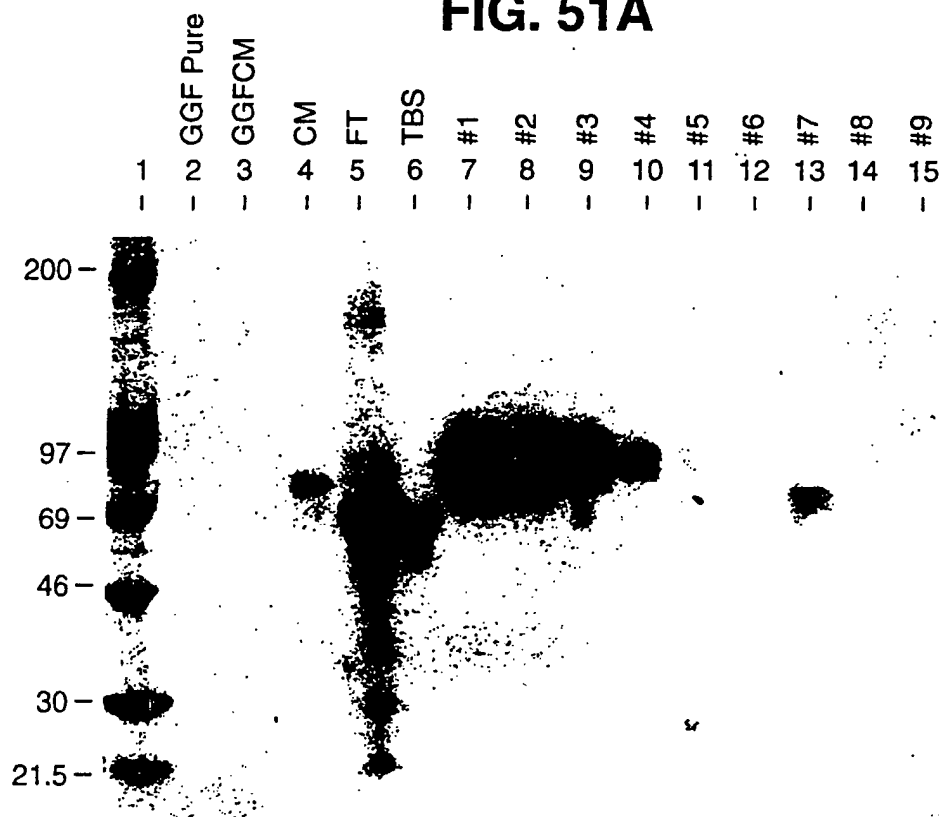
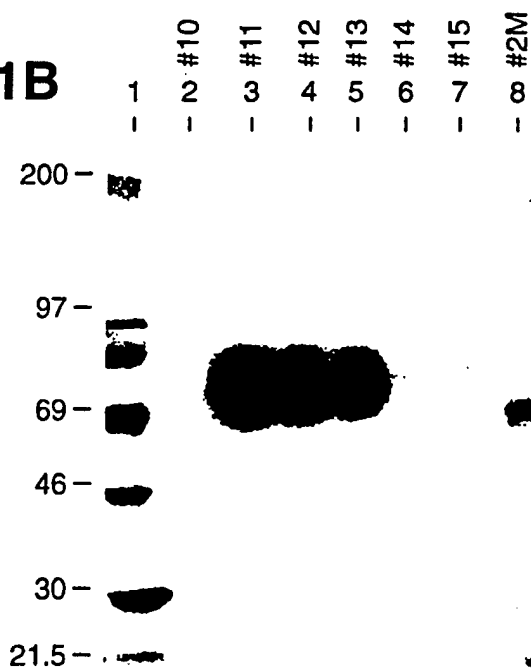
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FIG. 51

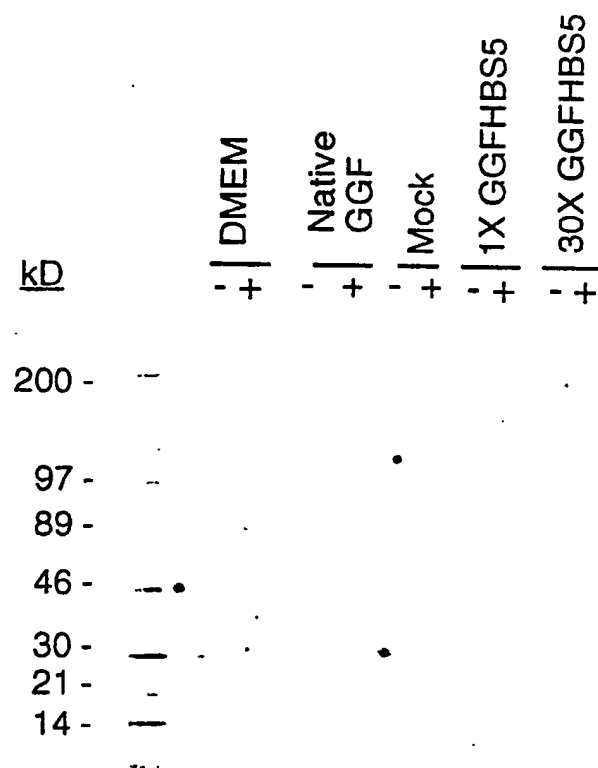




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**FIG. 51A****FIG. 51B**

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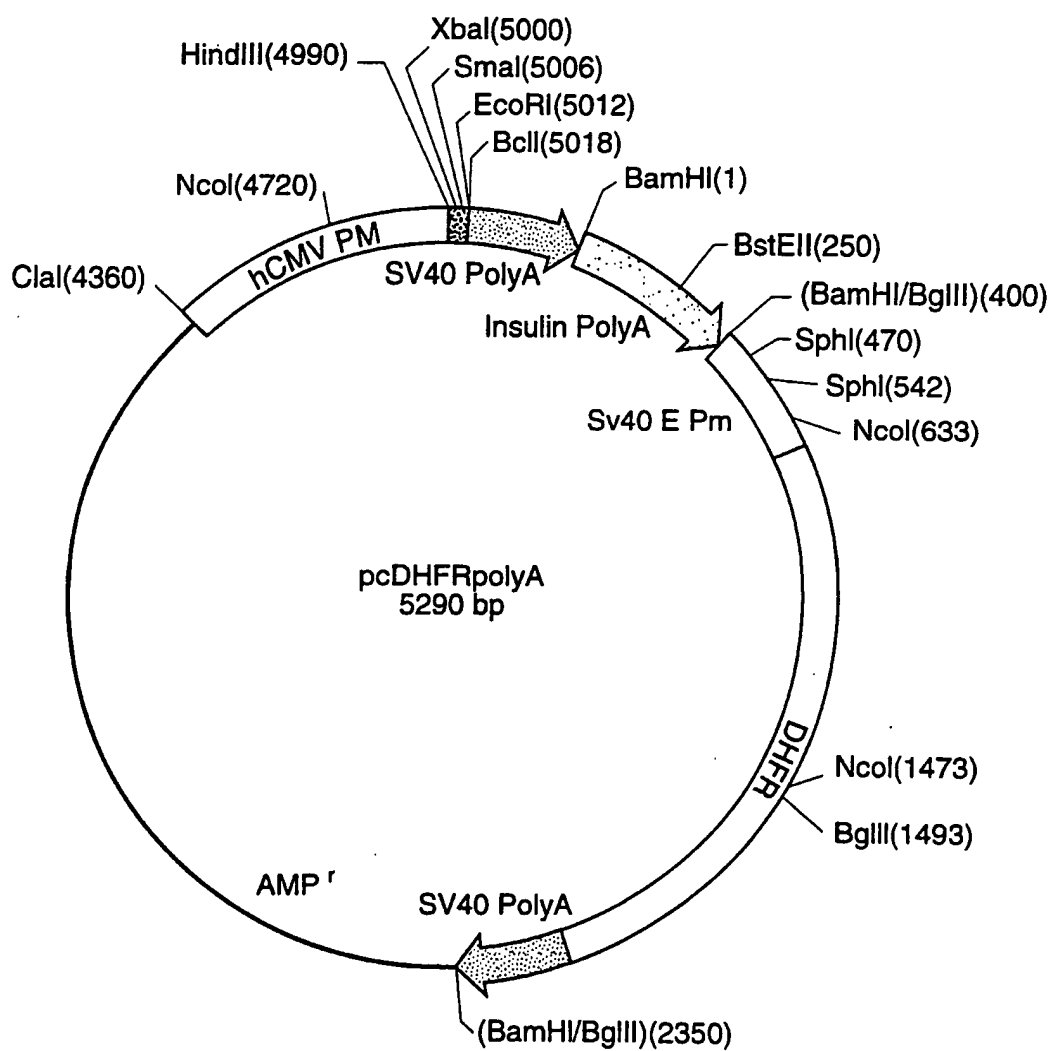
**FIG. 52**

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SEQ ID NO:170 GGFHBS5    1    MRMRAPRRSCRPGPRAQRPGSAARSSPPLPLLPLLLLTGAALAPGAAAGNEAAPAGAS  
                                II-8                 II-4  
61    VCYSSPPSVGSVQELAQRAAVVIEGKVHPORRQQGALDRKAAAAAGEAGAWGGDREPPAA  
                                O                                 II-1                 II-10  
121    GPRALGPAAEEPLLAANGTVPSWPTAPVPSAGEPEGEEAPYLKVHVQVAVKAGGLKKDSL  
                                II-3                                 II-2  
181    LTVRLGTWGHPAFPSCGRLKEDSR<sub>O</sub>YIFFMEPDANSTSRAPAAFRAFPPLETGRNLKKEV  
  
                                2                                 3  
241    SRVLCKRC.....▼  
SEQ ID NO: 171 GGFHFB1    1    O OMSEKRGKGKGGKKKERGSGKKKPESAAAGSQSP<sub>R</sub> R ALPPQLKEMKSQESAAGSK  
SEQ ID NO: 172 GGFBBP5    1    R K K G D VP GP R R V  
                                II-6    II-18    II-14    II-11    I-7, II-12,  
268    LVLRCETSSEYSSLRFKNFKNGNELNRKNKPQNIKIQKPKGKSELINKASLADSGEYMC<sub>\*</sub> III-13  
53  
53  
  
4    K S S S R S  
                                II-12                                 5  
328    KVISKLGNDASANITIVESN.....▼  
113    EIIITGMPASTEGAYVSSESPIRISVSTEGANTSSS<sub>T</sub> ATSTS  
113  
  
6    TTTGTSHLVKAEKEKTFCVNGGECFMVKDLNPSRYLCKPCPNEFTGDRCQNYVMASFYST<sub>\*</sub>  
354    A  
173  
173  
  
9  
413    STPF<sub>L</sub>SLPE\*  
232  
232

FIG. 53

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**FIG. 54**

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**FIG. 55**

1 MRWRRAPRRS GRPGPRAQRP GSAARSSPPL PLLPLLLLLG TAALAPGAAA  
51 GNEAAPAGAS VCYSSPPSVG SVQELAQRAA VVIEGKVHPQ RRQQGALDRK  
101 AAAAAGEAGA WGGDREPPAA GPRALGPPAE EPLLAANGTV PSWPTAPVPS  
151 AGEPEGEEAPY LVKVHQVWAV KAGGLKKDSL LTVRLGTWGH PAFPSCGRLK  
201 EDSRYIFFME PDANSTSRAP AAFRASFPPL ETGRNLKKEV SRVLCKRCAL  
251 PPQLKEMKSQ ESAAGSKLVL RCETSSEYSS LRFKWFKNGN ELNRKNKPQN  
301 IKIQKKPGKS ELRINKASLA DSGEYMCKVI SKLGNDSASA NITIVESNAT  
351 STSTTGTS HL VKCAEKEKTF CVNGGECFMV KDLSNPSRYL CKCPNEFTGD  
401 RCQNYVMASF YSTSTPFLSL PE\*

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/06846

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAPLUS, BIOSIS, WPIDS, MEDLINE, CA, N-GENESEQ 18, A-GENESEQ, SWISS-PROT31, PIR44  
search terms: neuregulin?, heregulin?, glia?, growth, factor#**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	Nature, Vol. 362, issued 25 March 1993, Marchionni et al, "Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system", pages 312-318, see entire document.	1-7, 10-18, 24, 25, 29-34 ----- 8, 9, 19-23, 26-28, 33
Y,P	Glia, Vol. 11, No. 2, issued June 1994, Raivich et al, "Pathophysiology of Glial Growth Factor Receptors", pages 129-146, see pages 134-135.	1-34
Y,P	US, A, 5,367,060 (VANDLEN ET AL.) 22 November 1994, see Column 39, Line 47 to Column 43, Line 13.	9,19,21-23, 26-28, 33

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 30 AUGUST 1995	Date of mailing of the international search report 25 SEP 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer STEPHEN GUCKER
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/06846

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/06846

### A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A61K 38/00, 38/16, 39/00, 39/395; C07H 21/04; C07K 1/00, 14/00; C12N 5/00, 15/09, 15/11, 15/12, 15/63; C12P 21/04; C12Q 1/00; G01N 33/53, 33/567

### A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

424/139.1; 435/4, 7.1, 7.2, 7.21, 69.1, 70.1, 70.3, 71.1, 240.1, 320.1; 514/2, 8, 12, 903; 530/324, 350, 387.1, 387.9, 395; 536/23.5; 930/10

### B. FIELDS SEARCHED

Minimum documentation searched

Classification System: U.S.

424/139.1; 435/4, 7.1, 7.2, 7.21, 69.1, 70.1, 70.3, 71.1, 240.1, 320.1; 514/2, 8, 12, 903; 530/324, 350, 387.1, 387.9, 395; 536/23.5; 930/10

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-4, 7, 10, 18, 29-32, and 34, are drawn to a single inventive concept of nucleic acids which encode for glial growth factors, the growth factors themselves, and methods of making the factors.

Group II, claim(s) 5, 6, 11-17, 24, and 25 drawn to methods of using glial growth factors as therapeutic agents.

Group III, claim(s) 8 and 20, drawn to methods to identify receptors for glial growth factors.

Group IV, claim(s) 9, 21, and 33, drawn to methods to inhibit binding of glial growth factors.

Group V, claim(s) 19, 22, 23, and 26-28, drawn to an antibody, and methods of making and using an antibody directed against glial growth factors.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Group II is drawn to methods of therapy utilizing glial growth factors that include in vivo methods and materials that do not share the special technical feature of nucleic acids that encode for glial growth factors of Group I.

Group III is drawn to methods of identifying receptors for glial growth factors that includes methods and materials that do not share the special technical feature of nucleic acids that encode for glial growth factors of Group I.

Group IV is drawn to methods of inhibiting glial growth factors that includes methods and materials that do not share the special technical feature of nucleic acids that encode for glial growth factors of Group I.

Group V is drawn to an antibody and a method of making and using the antibody which is a materially and functionally different and distinct protein than a glial growth factor and a glial growth factor is not required or used in the method of using the antibody. Furthermore, the antibody is not encoded by the special technical feature of nucleic acids that encode for a glial growth factor of Group I.

The nucleic acids and glial growth factors they encode of Group I, and the antibody of Group V have materially different structural and functional properties, each from the other. Thus the special technical features which identify each also distinguish each from the other.

Group I's method of making glial growth factors, Group II's method of using glial growth factors in vivo, Group III's method of identifying receptors, Group IV's method of inhibiting glial growth factors, and Group V's methods of using an antibody each use process steps and compositions that are materially different from the others and are unique to the group. Thus the special technical features that define each method distinguish them from the other.

Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.